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THE POTENTIAL OF MICROBIAL ECOLOGICAL INDICATORS TO GUIDE ECOSOPHISTICATED MANAGEMENT OF HYDROCARBON-CONTAMINATED SOILS

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ACADEMIC DISSERTATION

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ABSTRACT

Soil is an unrenewable natural resource on which we apply more and more pressures and demands. One of the main anthropogenic threats to soils, compromising their ability to provide us with the goods and ecosystem services we expect, is pollution. Hydrocarbons derived from crude and refined oils are the most prevalent soil contaminants, and have deleterious effects on not just biota but also the physicochemical properties of soils. Indigenous soil micro-organisms are the most functionally dynamic and responsive component of the soil ecosystem, and chronically in direct contact with the hydrophobic pollutants on the soil surfaces. Soil microbial variables could thus serve as an intrinsically relevant ecological indicator of soil quality, to be used as both technical and conceptual help in the ecological risk assessment of contaminated and remediated soils.

Two contrasting experiments – temporal monitoring after a single light contamination event in the greenhouse and on site spatial monitoring of heavy aged contamination – were designed to investigate soil microbial ecological responses to hydrocarbons, together with parallel changes in soil physicochemical and ecotoxicological properties. The aim was to identify quantitative or qualitative microbiological variables that would be practicable and broadly applicable for the assessment of the quality and restoration of oil-polluted soil. The major hurdle for the use of soil microbial analyses in the risk-assessment of oil-polluted sites is that soil bacteria commonly react on hydrocarbons as a beneficial substrate. This was found to lead to a positive response in the generally suggested soil quality indicators, even when the effect of oil on plants was toxic. Only in the case of heavy repeated contamination did the classical soil quality indicators accurately reflect the negative impacts on biota.

Due to various biological, physical and chemical processes leading to weathering of oil-contamination in soil, the contaminants become less bioavailable: their potentially toxic effects decrease faster than the total concentration quantified by exhaustive chemical extraction analyses. Indigenous hydrocarbon degrader bacteria, naturally present in any terrestrial environment, use specific mechanisms to improve access to the hydrocarbon molecules adsorbed on soil surfaces. Thus when contaminants are unavailable even to the specialised degraders, they should pose no hazard to other biota either. Changes in the ratio of hydrocarbon degraders to total microbes were detected to predictably indicate pollutant effects – when temporal or spatial decrease in this ratio ceases, the contaminants are no longer bioavailable.

Also qualitative characteristics of soil microbial communities reflect contamination and restoration. Bacterial community diversity was detected to decrease as a response to hydrocarbons, due to enrichment of degraders as

well as toxicity to other community members. Accordingly, stabilisation of community evenness, and community structure that reflects clean reference soil, indicate community recovery. If long-term temporal monitoring is difficult and appropriate clean reference soil unavailable, such comparison could possibly be based on DNA-based community analysis, reflecting past+present, and RNA-based community analysis, showing exclusively present conditions.

Microbial ecological indicators will never completely replace chemical oil analyses, but they are theoretically undeniably relevant and operationally practicable additional tools for ecological risk assessment. As such, they can guide ecologically informed and sustainable – ecosophisticated – management of oil-contaminated lands.

TIIVISTELMÄ

Maaperä on pitkälti uusiutumaton luonnonvara, jolle asetamme jatkuvasti enemmän odotuksia ja uhkia. Saastuminen on yksi tärkeimmistä maan toimintaa uhkaavista tekijöistä; saastuneella maaperällä on alentunut kyky tarjota maa- ja metsätaloustuotteita sekä ekosysteemipalveluita. Saastuttavista aineista yleisimpiä ovat öljyhiilivedyt (raakaöljy ja öljyjalosteet), jotka häiritsevät maaeliöstön lisäksi myös maaperän fysikokemiallisia ominaisuuksia. Mikro-organismit – bakteerit, arkit ja sienet – ovat dynaamisin ja maan toimintojen kannalta kenties olennaisin eliöluokka, joka on lisäksi jatkuvasti suorassa kontaktissa maapartikkelien pinnoilla olevien rasvaliukoisten kontaminanttien kanssa. Täten maan mikrobiologisia ominaisuuksia kuvaavat indikaattorit voisivat palvella sekä käsitteellisenä että teknisenä apuna öljyllä pilaantuneen maan kunnon ja kunnostumisen arvioinnissa.

Mikrobiekologisia vasteita öljyyn maassa tutkittiin kahdessa kokeessa: ensimmäisessä monitoroitiin kevyen kertasaastutuksen jälkeistä kehitystä kasvihuoneessa, toisessa analysoitiin muutoksia öljypitoisuuden suhteen raskaammin saastuneessa peltokohteessa. Maan fysikokemialliset ja ekotoksikologiset ominaisuudet palvelivat taustamuuttujina. Tavoitteena oli tunnistaa määrällisiä tai laadullisia mikrobimuuttujia, jotka olisivat helposti ja laajasti sovellettavissa öljyllä pilaantuneiden ja kunnostettujen maiden laadun arviointiin. Haasteena soveltuvien laatumittareiden löytämiselle oli, että maamikrobisto tyypillisesti reagoi hiilivetyihin ravintona eikä haitallisena aineena. Täten yleisesti ehdotetuissa maaperän laadun mikrobiologisissa indikaattoreissa nähtiin positiivinen vaste, vaikka öljysaastutus oli kasveille selvästi haitallista. Mikrobiston yleinen reaktio ilmensi öljyn haitallisia vaikutuksia ainoastaan raskaan toistuvan saastutuksen kohteena olleessa maassa.

Öljy säistyy maassa moninaisten biologisten, kemiallisten ja fysikaalisten prosessien vaikutuksesta, jolloin sen biosaatavuus heikkenee: öljyhiilivetyjen potentiaalisesti toksiset vaikutukset eliöstöön vähenevät nopeammin kuin kemiallisilla uutoilla mitattava kokonaismäärä. Missä tahansa maaympäristössä tavattavilla luontaisilla öljynhajottajabakteereilla on erityisiä keinoja edistää ravintona palvelevien öljyhiilivetyjen biosaatavuutta. Täten yleisen biosaatavuuden – josta toksisuuskin aiheutuu – pitäisi olla minimaalista siinä vaiheessa, kun öljy ei enää ole biosaatavaa erikoistuneille hajottajille. Erojen hajottajamikrobien ja kokonaismikrobiston määräsuhteissa havaittiin indikoivan ennustettavasti öljyn vaikutuksia. Kun tämä suhdeluku laskee ja lopulta tasaantuu alas öljyn määrän laskiessa, ei öljy enää ole biosaatavaa.

Myös maamikrobiston laadulliset ominaisuudet muuttuvat maan pilaantumisen ja puhdistumisen myötä. Bakteeriyhteisön monimuotoisuus

laski öljyn vaikutuksesta, johtuen sekä hajottajien suhteellisesta yleistymisestä että toksisesta vaikutuksesta muille bakteereille. Monimuotoisuuden nousu ja tasaantuminen ylös, samoin yleisesti kuin puhtaan verrokkimaan bakteeriyhteisöä muistuttava yhteisörakenne, indikoivat maamikrobiston toipumista öljykontaminaatiosta. Jos pitkäaikaisseuranta on mahdotonta ja vastaavaa puhdasta verrokkimaata ei ole saatavilla, voisi vertailun mahdollisesti tehdä DNA:n (mennyt+nykyinen yhteisö) sekä RNA:n (vain nykyinen yhteisö) erojen pohjalta.

Maamikrobiologiset testit eivät korvaa kemiallisia öljyhiilivety-määrityksiä, mutta ne tarjoavat teoreettisesti kiistämättömän relevantteja ja käyttökelpoisia lisätyökaluja ekologiseen riskinarviointiin. Täten mikrobiekologiset indikaattorit voivat ohjata öljyllä pilaantuneiden maiden ekologisesti tiedostavaa ja kestävää käyttöä.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their roman numerals:

I Mikkonen A., Kondo E., Lappi K., Wallenius K., Lindström K., Hartikainen H., Suominen L. 2011. Contaminant and plant-derived changes in soil chemical and microbiological indicators during fuel oil rhizoremediation with *Galega orientalis.*
Geoderma 160: 336-346.

II Mikkonen A., Lappi K., Wallenius K., Lindström K., Suominen L. 2011. Ecological inference on bacterial succession using curve-based community fingerprint data analysis, demonstrated with rhizoremediation experiment.
FEMS Microbiology Ecology 78: 604-616.

III Mikkonen A., Hakala K.P., Lappi K., Kondo E., Vaalama A., Suominen L. 2012. Changes in hydrocarbon groups, soil ecotoxicity and microbiology along horizontal and vertical contamination gradients in an old landfarming field for oil refinery waste.
Environmental Pollution 162: 374-380.

IV Mikkonen A., Santalahti M., Lappi K., Pulkkinen A.-M., Montonen L., Suominen L. Active bacterial and archaeal communities in aerobic and anaerobic soil layers in an old landfarming site for oil refinery waste.
Under review in Applied and Environmental Microbiology.

The contribution of the author to the papers:

Paper I The greenhouse experiment was designed and realised jointly by the authors. The contribution of the four first authors on the work was equal: Kaisa Lappi, Elina Kondo and Kaisa Wallenius performed the majority of the laboratory analyses, whereas Anu Mikkonen was responsible for the conclusive data analysis and writing of the manuscript. Anu Mikkonen was the corresponding author.

Paper II Anu Mikkonen set up and realised the community fingerprint analyses, and developed and performed the data analyses. She interpreted the results, wrote the manuscript and was the corresponding author.

Paper III The field experiment was designed and realised jointly by the authors. MSc students Anu Vaalama and Kati P. Hakala performed the hydrocarbon analyses and the majority of the basic soil analyses under the supervision of Elina Kondo. Kaisa Lappi was responsible for the *Vibrio fischeri* ecotoxicity test and the MPN enumeration. Anu Mikkonen interpreted the results, performed the statistical analyses, wrote the manuscript and was the corresponding author.

Paper IV Anu Mikkonen planned the experiment and supervised Minna Santalahti, who performed the community fingerprint analyses as part of her MSc thesis. Anu Mikkonen analysed the data, wrote the manuscript and was the corresponding author.

ABBREVIATIONS

BTEX	benzene, toluene, ethylbenzene, xylene
CCA	canonical correspondence analysis
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dw	dry weight
GC-FID	gas chromatograph with flame ionisation detector
ISO	International Organization for Standardization
LH-PCR	length heterogeneity polymerase chain reaction
mic_c	microbial biomass carbon
MPN	most probable number
OTU	operational taxonomic unit
PAH	polycyclic aromatic hydrocarbon
PCR	polymerase chain reaction
PLFA	phospholipid fatty acid
rDNA	gene encoding ribosomal RNA
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
TPH	total petroleum hydrocarbons
T-RFLP	terminal restriction fragment length polymorphism
TSEM	total solvent-extractable material
UCM	unresolved complex mixture
qPCR	quantitative polymerase chain reaction

1 INTRODUCTION

1.1 BACKGROUND AND MOTIVATION

In agriculture and forestry land is harnessed for the production of essential renewable goods: food, fodder, fuel and fibre. Global challenges and conflicts related to land use are expected to increase due to population growth and climate change. Supply of food must be secured for over 7 billion people on the planet, and the globally increasing consumption of meat and dairy products requires more feed and pasture lands. At the same time increasing areas of agricultural land is allocated to the production of biofuel plants instead of food and feed. Climate change, on the other hand, is feared to increase soil erosion and desertification rates, decreasing the size of productive land; intensifying urban sprawl will act similarly. Indisputably, never have we expected as much from land as we do now (Banwart, 2011; Foley et al., 2011).

In addition to the readily measurable agro-forestry products, soils also provide a number of ecosystem services, for which yields and production rates cannot be easily calculated. Soil-associated ecosystem services can be conceptualised and classified in multiple different ways. Supporting, provisioning, and regulating services, for example, include nutrient cycling, biodiversity resource and water quality regulation, respectively (Haygarth & Ritz, 2009). Many of these services can be tracked down to the activity of soil microbes: prokaryotic bacteria and archaea as well as eukaryotic fungi (Kibblewhite et al., 2008). Soil microbes occupy far less than 1% of overall surface on soil particles (Young and Crawford, 2004), but comprise the majority of the soil biomass (Winding et al., 2005); prokaryotes alone amount to astronomical numbers, up to 10^{10} cells per gram of soil (Torsvik et al., 1996). Moreover, micro-organisms are responsible for the final decomposition of all organic material and recycling of nutrients, estimated to account for more than 90% of the energy flow in soil (Nannipieri et al., 2003; Winding et al., 2005). Although we are only beginning to understand the extent and intricacy of the various ecosystem services provisioned by soil and its microbes, it is clear that they are indispensible. No sum of euros can compensate for the essential turnover of organic matter and cycling of elements – for it is microbes, not money, that make the world go round.

We demand more and more from the decreasing land area, but simultaneously we pose soils with a multitude of anthropogenic threats that can severely decrease the quality of soil and compromise its ability to provide the goods and services we expect from it (Haygarth & Ritz, 2009). The hazard of soil degradation is underpinned by the fact that soil is a by and large unrenewable natural resource, formed through very slow physicochemical and biological processes that transform rocks into clay and

biogenic material into recalcitrant soil organic matter (Banwart, 2011). The Thematic Strategy for Soil Protection (Commission of the European Communities, 2006) emphasises contamination as one of the major indicators of soil degradation. In Europe there are estimated to be half a million contaminated sites in need of risk assessment and remediation; for Finland the figure is 20 000, and total number for England and Wales may sum up to 100 000 (Brassington et al., 2007). When pollutant concentrations exceeding guidance values are detected and/or a risk of human exposure is identified, the common solution is to excavate and dispose the contaminated soil masses as harmful waste (Sorvali, 2010). In sparsely populated areas like Finland this is still feasible – however, the increasing costs due to rising fuel price, and stricter regulatory demands expected from the European Union Soil Directive, will make such a quick fix less appealing.

The most common contaminant class are various hydrocarbons that originate from crude oil or refined oil products (European Environment Agency, 2007). Most of these compounds are biodegradable; micro-organisms seem to be able to degrade and utilise practically any organic molecule, and associated metabolic reactions have been studied intensively for decades (Van Hamme et al., 2003; Robertson et al., 2007). The degrader microbes can be found in any environment contaminated with hydrocarbons, and their natural catabolism is harnessed in bioremediation, biologically mediated clean-up of contaminated material (Brassington et al., 2007). Despite the promise of bioremediation as an easy solution to pollution, the thresholds of contaminant degradation in soil are still not understood well enough, often resulting in unpredictability of the process and unsatisfactory results (Brassington et al., 2007; Robertson et al., 2007). Regarding the extreme complexity of all the three factors – soil, its microbes, and hydrocarbons – it could well be claimed that biodegradation of oil in soil is one of the most complicated processes man has ever attempted to engineer.

Even though oil may benefit specialised degrader organisms, it acts as a harmful pollutant on the majority of biota. Risk assessment for environmental pollution is currently focused on estimating human exposure (Brassington et al., 2007); very little attention is given to the ecological risks of soil contamination. If we see no intrinsic value in unspoilt nature *per se*, it might be asked why care about ecological aspects at a human-impacted site that is no nature reserve and inhabits no endangered species. However, it is arguably sensible to ask whether the contaminated site can, before or after remediation activities, provide those ecosystem services that we still expect from it. With this regard, the essential questions are the bioavailability of the pollutant *in situ* (where it is) and its chronic effects on the intrinsic soil biota: direct as well as indirect effects, e.g., through changes in soil physicochemical properties. Monitoring soil microbes, which govern many of the functions behind the ecosystem services, could thus provide valuable and relevant information on the ecological status of the contaminated site. Despite the great potential of the microbial ecological approach for the evaluation of soil

contamination, inconsistent responses or even the insensitivity of microbial parameters to soil hydrocarbons have been reported (Semple et al., 2003; Paton et al., 2005). Earlier results on the subject area seem to have triggered as many new questions as they have answered.

1.2 OUTLINE AND OBJECTIVE OF THE WORK

The general aim of this thesis was to identify microbial ecological variables that would be suitable indicators for soil contamination and restoration. The strategy was to utilise a multidisciplinary approach: microbial responses to contamination and restoration were monitored in close association with traditional soil physicochemical properties as well as contaminant chemistry. As microbes are the most dynamic and responsive component of any functional soil, I hypothesised that the appropriate microbial indicators would react on both contamination and restoration more rapidly and sensitively than soil physicochemical characteristics.

Two contrasting case studies were designed. The first case (Figures 1 & 2; Papers I & II) was a greenhouse experiment, where the effects and degradation of light fresh contamination were examined by temporal monitoring. The second case (Figure 3; Papers III & IV) assessed chronic impacts of heavy weathered contamination through spatial monitoring *in situ* on an old landfarming field.

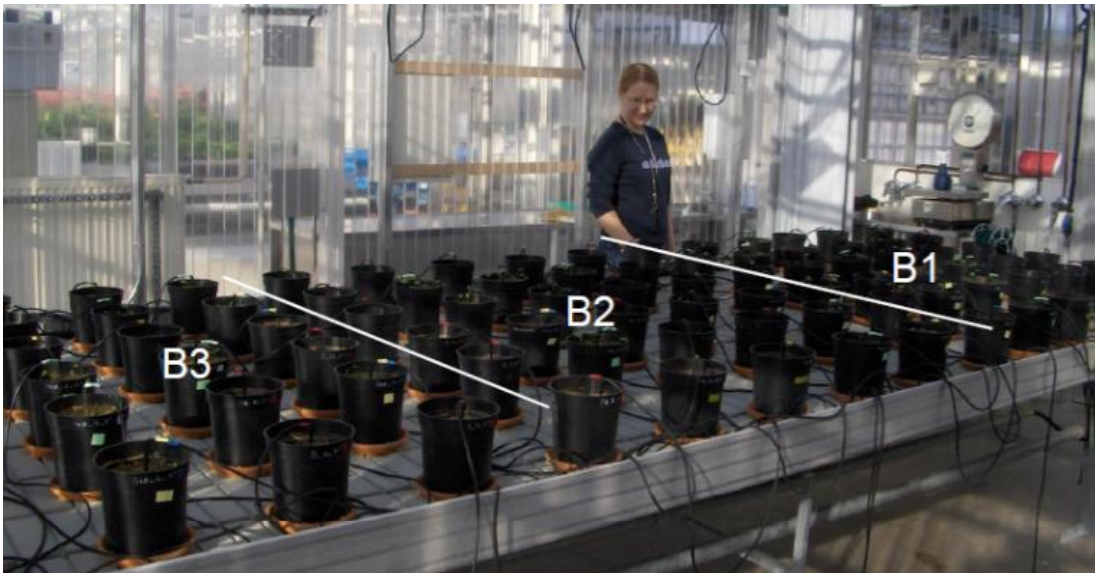

Figure 1 Setup of the first case, a 21-week greenhouse experiment. B1, B2 and B3 refer to blocks, each of which contained one replicate pot for each treatment and each sampling week. The places of the pots within each block were randomised weekly. Person in the photo: Kaisa Lappi.

Figure 2 The set of pots removed from the greenhouse experiment at each of the 10 sampling times for soil sieving, mixing and sample storage. Treatments from left to right: Vegetated, ContaminatedVegetated, Contaminated, ContaminatedSterilised.

Figure 3 Landfarming field at the Kilpilahti industrial site in Porvoo, southern Finland (second case; photo taken 1.10.2009). Notice the contamination gradient from left to right, visualised by slower development of vegetation after ploughing. The fenced study area, where all the samples have been taken from, was ploughed for the last time in spring 2007.

In both experiments we analysed quantitative and qualitative microbial ecological variables, as summarised in table 1. A detailed description of each method can be found in the paper which first mentions it.

Table 1 *Experimental setup of the two case studies and the analytical methods used in the four original publications.*

		Paper I	Paper II	Paper III	Paper IV
Case study	Contaminant	Freshly added fuel oil (TPH 3 g/kg)		Weathered crude oil (TPH surf. 3-7, subsurf. 5-46 g/kg)	
	Location/ study design	Destructive greenhouse experiment		Analysis of *in situ* gradients at oil refinery waste landfarming field	
	Source of variation	Treatments (contaminated-vegetated, contaminated, vegetated) Temporal change (weeks 0-21)		Horizontal contaminant gradient Vertical contaminant gradient	
Background data	Soil analyses	C, N, pH, dw, plant dw and N%	-	C, N, P, pH, WHC, EC, CEC, Na, dw	C, N, pH, EC, dw
	Oil analyses	TPH, *n*-alkanes vs. iso-alkanes	-	TPH, TSEM, aliphatics, aromatics, polars, *Vibrio fischeri* ecotoxicity test	TPH, TSEM
Microbiological soil analyses	Quantitative analyses	DNA quantity, fuel oil MPN, 10 enzymatic activities	-	Basal respiration, fumigation extraction, fuel oil MPN	Fumigation extraction, DNA & RNA quantity
	Qualitative community analyses	-	Bacterial community LH-PCR (DNA), cloning and sequencing of responding classes	Bacterial community LH-PCR (DNA)	Bacterial and archaeal community LH-PCR (DNA & RNA), cloning and sequencing of dominant classes

TPH = total petroleum hydrocarbons (nonpolar C_{10}-C_{40} hydrocarbons)

dw = dry weight, to which all concentrations are normalised

MPN = most probable number enumeration

LH-PCR = length heterogeneity polymerase chain reaction

WHC = water holding capacity, EC = electrical conductivity, CEC = cation exchange capacity

TSEM = total solvent-extractable material (total hydrocarbons)

The following chapters describe those aspects of the original papers that are relevant to the topic of this synthesis – for detailed information I refer to the tables and figures in the original publications found at the end of this thesis compilation. Chapters 2 Oil in soil and 3 Microbial ecological responses to organic contaminants in soil briefly describe and justify the chemical and microbiological methods used, as well as present the main results. Discussion can be found in chapter 4; here I emphasize the interpretation of microbiological variables in the context of risk assessment for soil hydrocarbon pollution. In chapter 5 Conclusions I summarise the major outcomes of the work and evaluate their applicability.

2 OIL IN SOIL

Soil is the most complicated biomaterial on the planet.

The striking claim above, stated by Young and Crawford (2005), only begins to describe the complexity of the dirt below our feet. Soil is comprised of three phases: solid, gaseous and aqueous. Due to the vast size range of the mineral particles from sand (50-2000 mm) to clay (<2 µm), these phases form fractal-like structures. These non-random porous structures are maintained and modified by soil-dwelling organisms; especially important is the role of microbes that degrade organic compounds, which also show great heterogeneity in quantity, quality and distribution (Young & Crawford, 2005; Or et al., 2007).

Oil-derived hydrocarbons are one class of organic substrates for soil microbes. Different hydrocarbons vary in hydrophobicity, but are collectively characterised by poor solubility in water. This insolubility by definition denotes that the distribution of oil in structured soil will be heterogeneous and increase the spatial variability of soil characteristics, both horizontally and vertically (McAllister & Semple, 2010). Experimental consequences are that one has to either: 1) study structured contaminated soil in which the large variability makes inference on general trends extremely difficult, 2) apply the contaminant to structured soil with organic solvent that improves spreading but is likely to kill the majority of soil organisms, or 3) eliminate soil structure by mixing, both upon contamination and sampling, which destroys one of the most characteristic properties of soil, but makes comparisons and assessment of general trends possible. Like the majority of earlier studies on soil hydrocarbon contamination, also this thesis work utilises the third approach: careful sieving and mixing of each sample to enable linking data from multiple background analyses to the results of oil and microbial analyses.

2.1 PETROLEUM HYDROCARBONS

Petroleum hydrocarbon contaminants include aliphatic, monoaromatic (BTEX, i.e., benzene, toluene, ethylbenzene, xylene) and polyaromatic hydrocarbon (PAH) compounds which can be found in crude and refined oils in different ratios. Besides C and H, crude oil also contains traces of S and N in heterocyclic molecules (Van Hamme et al., 2003). Hydrocarbons, typically originating from subsurface oil reservoirs, can enter the environment naturally from, for example, sea floor seepages. However, significant concentrations in soil are usually of anthropogenic origin, from gradual

build-up at petroleum stations or industrial sites, single spill incidents or deliberate spreading.

Crude and refined oils are extremely complex mixtures that contain hundreds of different compounds, with widely differing chemical properties (Wang and Fingas, 1997; Serrano et al., 2008). Based on the boiling point range, both crude and refined oil can be characterised as light (comprised of small molecular weight compounds that gasify at lower temperatures) or heavy (containing larger molecules that need extreme temperatures to volatilise). Also the age of the oil, i.e., for how long it has been exposed to abiotic and biotic dissipation processes, affects the heaviness of the analysable remaining fraction. Lighter compounds (short-chain alkanes and BTEX) are more prone to evaporation, leaching and degradation, making aged or weathered oils relatively heavier (Alexander, 1995; Brassington et al., 2007). A practical way to examine both the original composition and the degree of weathering of oil in an environmental sample is the analysis of total petroleum hydrocarbons (TPH), which quantifies solvent-extractable non-polar C_{10}-C_{40} compounds using a gas chromatograph equipped with a flame ionisation detector (GC-FID) (ISO 16703:2004). The abundance and height of the discrete peaks (aliphatics) and the relative size and location of the hump (unresolved complex mixture, UCM) are the characteristic GC-FID chromatogram features (Figures 4 & 5 – compiled from Papers I & III, respectively). The ratio of readily degradable straight-chain *n*-alkanes to more recalcitrant branched iso-alkanes has also been used as an indicator of biodegradation (Wang and Fingas, 1995). However, during the degradation of fuel oil in a greenhouse, the ratios (C_{17}/pristane and C_{18}/phytane) decreased expectedly only for the first week and then turned to steep increase (Paper I). This indicator seems thus unsuited for monitoring the biodegradation of light hydrocarbon contamination in near-optimal conditions.

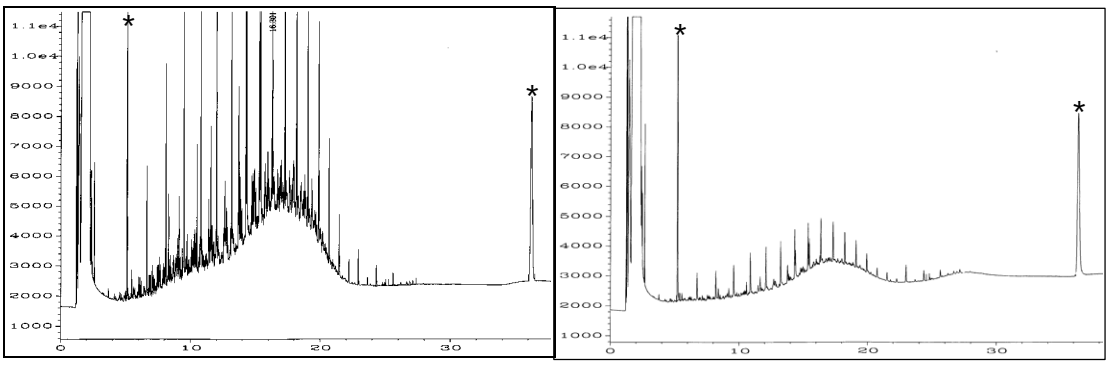

Figure 4 TPH chromatograms (GC-FID) from non-vegetated greenhouse soil contaminated with fuel oil 4 days after contamination (1.7 g/kg; left) and after 21 weeks of biodegradation (0.4 g/kg; right). The high C_{10} and C_{40} peaks, marked with asterisks, limit the analytical range.

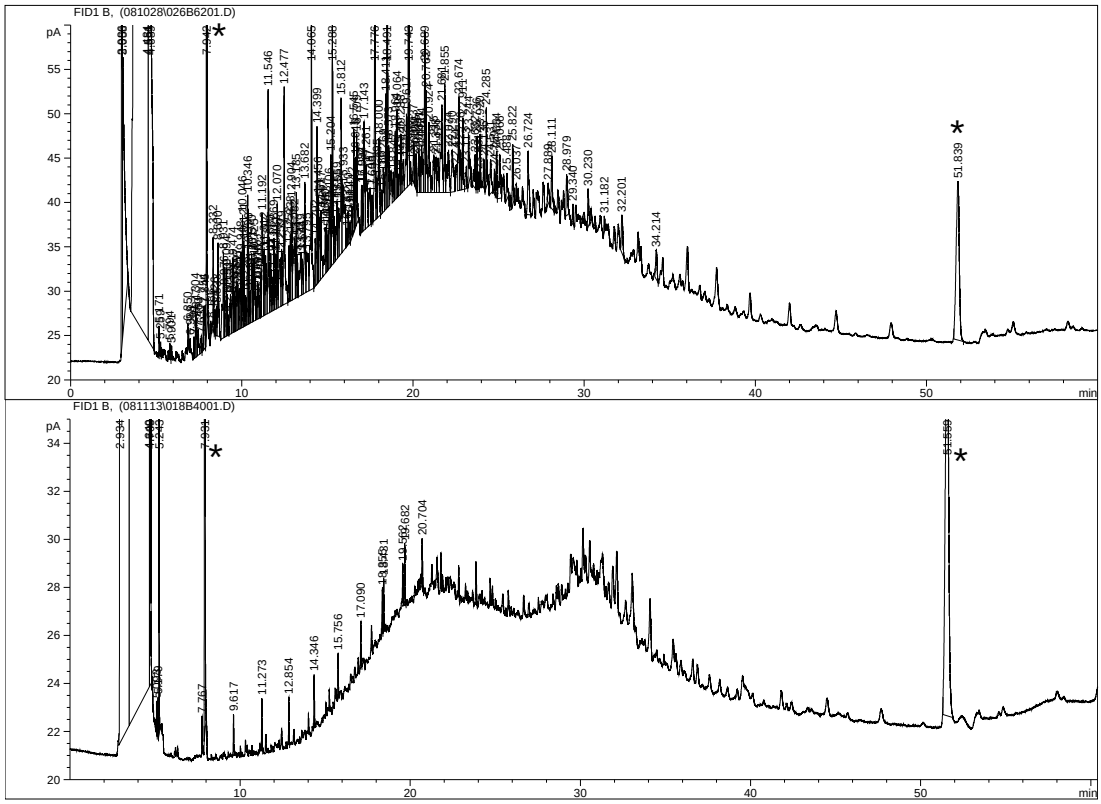

Figure 5 TPH chromatograms (GC-FID) from landfarming field soil contaminated with crude oil, practically non-weathered oil at the 40-60 cm layer (46.0 g/kg; above) and heavily weathered oil at the 0-20 cm layer (6.8 g/kg; below) (note the different scales of the y-axes: 60 pA in the upper figure and 35 pA in the lower).

Aliphatic hydrocarbons are generally more susceptible to biodegradation than aromatic compounds with a chemically stable benzene ring (Van Hamme et al., 2003). Thus also the aliphatics/aromatics ratio can serve as a biodegradation indicator. Advanced analytics, i.e., the attachment of a GC to a mass spectrometer, is required to identify and quantify individual PAH compounds (Wang and Fingas, 1995). However, the relative abundances of aliphatics and aromatics can be estimated simply by fractionating the hydrocarbon crude extract using a silica column: aliphatics elute with the original non-polar solvent heptane, aromatics with the more polar heptane:dichloromethane mixture (Paper III, fig. S1; Wang & Fingas, 1997). These fractions can then be quantified either gravimetrically (by weighing) or by GC-FID, which is more sensitive but leaves high molecular weight compounds undetected. Results of Paper III, fig. 1 demonstrate the feasibility of the fractionation: aromatics equalled to or outweighed aliphatics in the weathered oil in topsoil (0-20 cm) whereas less degraded oil in deeper anaerobic layers (40-60 cm) still contained relatively more aliphatics. Surprisingly, in the case of heavily weathered crude oil contamination, concentrations of both aliphatics and aromatics measured by GC-FID superseded TPH concentration (Paper III, table 1). According to the abstract, “ISO 16703:2004 is applicable to the determination of all

hydrocarbons with a boiling range of 175 °C to 525 °C, *n*-alkanes from $C_{10}H_{22}$ to $C_{40}H_{82}$, isoalkanes, cycloalkanes, alkylbenzenes, alkylnaphthalenes and polycyclic aromatic compounds, provided that they are not absorbed on the specified column during the clean-up procedure." However, apparently purification of the crude extract by Florisil magnesium silicate, characteristic of this ISO standard method, traps much more hydrocarbons than silica. This finding raises the question of how well suited TPH analysis alone is "for the quantitative determination of the mineral oil (hydrocarbon) content in field-moist soil samples" (ISO 16703:2004). The disparity was even more striking, nearly by an order of magnitude, when comparing TPH to total hydrocarbons gravimetrically quantified from the crude extract - although it could be deduced from the reverse direction of vegetation and contamination gradients that the extracted and weighed material truly was of oil-waste origin and not plant-derived (Paper III). The majority of the difference was explained by polar compounds, eluted from the silica column with methanol and quantified gravimetrically. However, also non-GC-FID-resolvable high molecular weight hydrocarbons that may have formed from the polar hydrocarbon metabolites (Robertson et al., 2007) amounted to approximately a quarter of the total hydrocarbons (Paper III, fig. 1). Our findings provide the first strong experimental evidence suggesting that TPH is unsuitable for monitoring biodegradation of weathered crude oil-derived hydrocarbons in soil, if the aim is hydrocarbon mineralisation. For sites or soil masses with weathered contamination, evaluation of remediation success should not be based on mere TPH measurement but a more complex assessment of reduction of risk (Brassington et al., 2007).

First-order reaction kinetics, generally assumed for hydrocarbon biodegradation, lead to a hockey stick-shaped degradation curve (Paper I, fig. 2a). No nill level is achieved, but a recalcitrant non-degradable fraction of some size typically remains (Semple et al., 2003). With light fuel oil contamination the degradation levelled off at a concentration so small that the soil would be legislatively regarded clean (from initially applied 0.3% to 0.03%). However, more carbon remained in the soil (approx. 0.1%), meaning that the dissipated oil was not completely mineralised. It is difficult to ascertain whether the remaining carbon was also in this case as polar intermediate metabolites: hydrocarbons that are not completely mineralised but partly oxidised to forms undetectable by TPH analysis. Although the formation and behaviour of such metabolites in soil has been altogether very little studied, they are generally regarded to pose a risk due to assumedly higher mobility and toxicity (Rončević et al., 2005; Brassington 2007; Robertson et al., 2007). The other explanation for the remaining carbon in Paper I was that it was assimilated to the biomass of the degrader microbes, likely in harmless forms.

The aerobic degradation of organic material (respiration) generally tends to acidify soils with little buffer capacity (Standing & Killham, 2007), typical of Finnish lands poor in carbonate minerals. Biodegradation of hydrocarbons

should make no exception to this; oil contamination and bioremediation lower soil pH (Aislabie et al., 2006), as was also observed in Paper III, table 2 assessing the long-term contaminated site. Unexpectedly, in the greenhouse experiment where pH was monitored at short intervals, it was observed to increase during the most active first weeks of fuel oil biodegradation (Paper I, fig. 2B). Possible explanation would be reduced redox potential due to anoxic conditions, but the rapidity of hydrocarbon degradation argues for aerobic conditions. This surprising result demonstrates how we still cannot predict the effects of oil and its degradation in soils with certainty. However, what can be confidently stated is that the hydrophobic compounds must be bioavailable to the organisms attacking them to be degraded – the next chapter will discuss this limitation.

2.2 BIOAVAILABILITY OF ORGANIC CONTAMINANTS

Exhaustive extractions with solvents, described above, aim at quantifying the total concentration of contaminants that remain in the soil. However, the amount of hydrocarbons extracted by hydrophobic organic solvents from soil is generally higher than the concentration experienced by soil biota (Semple et al., 2003; Paton et al., 2005). The bioavailability of hydrocarbons in soil is a prerequisite for their microbiological degradation – as well as for toxic effects on soil organisms at any trophic level (McAllister & Semple, 2010; Grotenhuis & Rijnaarts, 2011). Oils consist mostly of alkanes, which are not especially toxic compared to most other organic contaminants (Tecon & van der Meer, 2008). However, the typical concentration of alkanes in contaminated soil usually by far exceeds the concentration of other contaminant classes, e.g., chlorinated hydrocarbons or heavy metals. Alkanes are extremely hydrophobic, and although the solubility of aromatic compounds in water is somewhat higher, the mass of hydrocarbon contaminants in soil is on the solid phase surfaces, not dissolved to soil water (Semple et al., 2003; Paton et al., 2005). Nevertheless, also hydrocarbons adsorbed on surfaces can be accessible to degrader microbes (Siciliano & Roy, 1999; McAllister & Semple, 2010).

The size of the bioavailable fraction is related to the total contaminant concentration (Paton et al., 2005), and thus biodegradation of oil is bound to decrease the availability of oil to biota. Besides biological activity, also multiple abiotic reactions alter and decrease the bioavailable fraction of oil as it enters soil (Alexander, 1995); collectively, these processes cause oil weathering or aging. Lightest hydrocarbons are directly volatilised and some may be prone to leaching (McAllister & Semple, 2010). Regardless of how light or heavy the oil is, the majority of it is always trapped from the liquid phase to the soil matrix in sequential processes. The initial reaction, the gravitation of oil to thin films on surfaces, is rather rapid unless the oil amount is so large that it remains as a phase of its own (non-aqueous phase

liquid, NAPL; Grotenhuis & Rijnaarts, 2011). However, the sequestering of oil to soil solids proceeds progressively (McAllister & Semple, 2010). Besides adsorption onto surfaces of minerals and condensed organic matter, oil diffuses into pores and fractures on these surfaces and is absorbed by flexible organic matter (MacLeod et al., 2001; Loibner et al., 2006; McAllister & Semple, 2010). Due to hydrophobic interactions, organic matter is the preferred matrix that dominates sorption if its abundance in soil exceeds 0.1% (MacLeod et al., 2001; McAllister & Semple, 2010; Grotenhuis & Rijnaarts, 2011). Due to the strong capacity of the organic solid phase to retain hydrophobic contaminants, the guidance values (limits of unacceptable concentration) for some organic pollutants have even been suggested to be adjusted by soil organic matter (SOM) content (Van-Camp et al., 2004). In the case of oil contaminants this might not be sensible, since their concentrations can often be high enough to form a significant proportion of SOM, even exceeding the plant-derived fraction (Paper III). Also a larger surface area of soil, i.e., higher clay content, may reduce negative impacts of oil due to a smaller proportion of surface impacted (McAllister & Semple, 2010). Interestingly, weathered heavy oil was found to clump smaller mineral particles together, interfering with particle size analysis by reducing the observed clay content (Paper III). Both findings stress that especially weathered heavy oil can severely disturb even the basic soil analyses, which are a prerequisite for any terrestrial study.

Various non-exhaustive extraction methods have been developed and evaluated to estimate the concentration of readily accessible pollutants (Semple et al., 2003; Paton et al., 2005). However, besides total concentration and sorption to the soil matrix, the bioavailability of hydrocarbons depends on the defined receptor organism (Paton et al., 2005). Thus bioavailability is commonly assessed through the responses of biological receptors in bioassays: ecotoxicity tests and biosensor assays (Fränzle, 2006; Tecon & van der Meer, 2008).

Plants are often used in soil ecotoxicity testing; they are a relevant receptor because the production of agro-forestry goods is based on the ability of soil to support vegetation. Leguminous fodder galega (*Galega orientalis*) showed a clear negative response to light fuel oil in the greenhouse experiment (Paper I, fig. 1), although this plant has been earlier reported to be relatively resistant against hydrocarbon contamination (Suominen et al., 2000). The difference in plant biomass between contaminated and uncontaminated soil disappeared only at the end of the 21-week experiment when the soil was already practically clean. Deleterious effects on plant biomass development were observed also with the native weeds growing at the landfarming field (Paper III). Between the ploughings twice during growing season, plants only grew at the less contaminated end of the horizontal hydrocarbon gradient. Anyhow, dense vegetation, dominated by grasses and mosses, developed on all the experimental plots after the regular

tillage was stopped, indicating that the plant growth was not inhibited but only retarded (Figure 3).

Besides plant tests that can be carried out with soil *per se*, the majority of bioassays are aquatic, based on extracts or slurries of the contaminated soil. The kinetic "Flash" version of the *Vibrio fischeri* photobacterium test (ISO 21338:2010) was deemed especially promising for the assessment of the ecotoxicity of the weathered hydrocarbons at the landfarming field; the test is carried out in adjusted pH and salinity, and should therefore not respond to acidity and sodium increasing along with oil content in the horizontal contaminant gradient. Although the test was performed with soil slurries up to a suspension ratio of 1/10, toxicity was only observed for the most contaminated surface soil plot (Paper III, fig. 2). Subsurface samples were not measured because the respiratory activity of *V. fischeri* requires well aerated conditions, which would have likely been compromised with severely contaminated anaerobic samples. According to ISO 21338:2010, samples with a high oxygen demand may be inhibitory, but extended aeration pretreatment would surely have affected the quantity and quality of hydrocarbon contaminants.

Even if the induction of light production caused by soil properties likely competed with pollutant-derived toxicity and contributed to the insensitivity of the test, these results showcase the major problem of aqueous ecotoxicity tests with hydrophobic contaminants: the toxic pollutants are on the soil surfaces and not in direct contact with the planctonic marine test organisms in the aqueous phase. Tests for soil extracts have also earlier been reported insensitive (MacLeod et al., 2001), but the problem with the matrix effect and hydrophobicity probably extends to all aqueous ecotoxicity tests, which cannot take into account the matrix effect essential to understanding toxicity in soil (Alexander, 1995; Fränzle, 2006). The same disadvantage may apply to genetically modified biosensor strains extensively developed and tested during the last decade; according to Tecon & van der Meer (2008), detection of long-chain alkanes $>C_{10}$ with specific biosensors has proven difficult due to their extremely low solubility in water (~10 nM). Moreover, biosensors are usually developed to respond to specific compounds or classes and might not be able to sufficiently signal the availability and toxicity of mixed contamination, which is the default in the case of oil-contaminated soils.

3 MICROBIAL ECOLOGICAL RESPONSES TO ORGANIC CONTAMINANTS IN SOIL

When evaluating the toxicity of a contaminant in soil, one should carefully consider the relevance of the test organism, exposure method and test endpoint. The challenges related to aquatic tests and test organisms were discussed above. In addition, when measuring the acute response of a few selected test organisms actively exposed to the added pollutant, it must be born in mind that some species or trophic levels may be more tolerant to contaminants, and others significantly more sensitive (Winding et al., 2005; Fränzle, 2006). This so-called species sensitivity distribution, as well as uncertainty from limited data, is typically accounted for by dividing the experimentally detected "safe concentrations" with application factors, e.g., 10, 100 or 1000. In some cases this has resulted in ecotoxicological soil quality criteria that are below contaminant natural background concentrations (Scott-Fordsmand & Jensen, 2002).

Assessing the ecological risk from contamination by passive ecotoxicity tests, with the indigenous microbiota of the polluted soil, might solve a majority of these problems. Such organisms are undeniably relevant for the functioning of soil and the ecosystem (MacLeod et al., 2001). They are directly exposed to contaminants in soil, as both microbes and hydrophobic pollutants are located on surfaces (Siciliano & Roy, 1999; Or et al., 2007; McAllister & Semple, 2010). Moreover, due to their large surface/volume ratio, especially prokaryotes react to environmental changes more sensitively than larges organisms (Winding et al., 2005). Passive *in situ* ecotoxicity tests also take into account chronic exposure and additive effects of multiple stressors: chemical, physical and biological (Paton et al., 2005; Fränzle, 2006). Due to the aforementioned points, Winding et al. (2005) argued for the relevance of estimating environmental disturbance at the microbiological trophic level. In fact, soil prokaryotes alone represent several trophic levels: primary producers (autotrophs), and primary consumers as well as decomposers (heterotrophs). The challenge in monitoring the responses of *in situ* micro-organisms is that they cannot be monitored directly without disturbance. However, sacrificial sampling can reveal snapshots of the soil microbial ecological status, which should be linked to soil physicochemical properties for inference of spatial (and temporal) changes. Typically contaminant concentration-dependent or otherwise predictable response of the microbial variables is expected. Nevertheless, Siciliano & Roy (1999) warned that the basic assumptions of toxicology, such as concentration-dependent response, may not always be directly applicable in the soil environment.

Unlike ecologists studying macro-organisms, soil microbial ecologists can seldom restrict their surveys to one or a few species. The ecology of the

underground is less well characterised than any other habitat on Earth (Sugden et al., 2004). The vast diversity of soil prokaryotes (Gans et al., 2005; Delmont et al., 2011b), debated microbial species definition and unknown higher taxa (Mora et al., 2011), as well as functional redundancy (Allison & Martiny, 2008), pose great theoretical challenges to research. Accordingly, community level measurements of wider groups or functions are generally regarded ecologically more relevant than narrow focus on specific species or genes (Schloter et al., 2003; Fränzle, 2006).

Microbial ecological composite measures are typically either quantitative or qualitative by nature; the next chapters describe the response of soil microbiota to hydrocarbon contamination under these two broad categories. Focus is on the results of the original publications (Papers I-IV), although analytical caveats and some alternative methods commonly used in the analysis of contaminated soils are also briefly discussed. Due to the selected comparative experimental strategy explained in the beginning of chapter 2, *in situ* visualisation methods will not be discussed here.

3.1 QUANTITATIVE MEASURES

Quantitative microbial ecological measures produce a single numerical value for a microbiological count or process rate. Typically the focus is on bacteria; they are often the most numerous micro-organisms in disturbed soils and include well-characterised degrader taxa (Van Hamme et al., 2003). In addition, basic knowledge on and analytical methods for soil bacteria are more advanced compared to archaea and fungi, which are generally more resistant against cultivation attempts. However, fungal activity is accounted for in many gross biomass and activity measures, and their relative importance is generally higher the more acidic the soil is (Rousk et al., 2010). Archaea, on the other hand, seem to be specialised in ecological niches with severe energy stress (low amount or thermodynamically unfavourable quality of substrates and/or electron acceptors; Valentine, 2007). The results of this life strategy are generally lower biomass and slower responses.

As stressed by MacLeod et al. (2001), enumeration methods only enumerate the faction that can grow, be otherwise amplified or detected in the conditions used. Culture-dependent methods, especially, rarely quantitatively reflect *in situ* conditions. However, the fact that only a part of the target pool is successfully quantified does not necessarily impair comparative studies (Griffiths et al., 2003). Unfortunately, the extent and quality of culture bias are seldom known and these may significantly differ for different samples. Thus care is needed when interpreting cultivation-based results.

3.1.1 MICROBIAL COUNTS

Regardless of the fact that hydrocarbons are considered toxic environmental pollutants, oil contamination typically increases bacterial numbers in soil. A single contamination with fuel oil to 0.3 g/kg (tenfold to clean soil reference value) tripled the concentration of soil DNA that was used as a proxy of microbial biomass (Paper I, fig. 2c). The response in contaminated soils did not depend on the vegetation status, meaning that the DNA was of microbial origin and not root-derived. However, a similar but delayed increase was observed in the non-contaminated soil in association with the growth of the legume fodder galega. Thus appropriate reference treatments are required to distinguish the actual oil-effect from responses to, e.g., mixing and thawing, which also generally release substrates for growth (Ollivier et al., 2011).

Oil-induced microbial growth, either instantly or after a rehabilitation period, could be monitored with a variety of comparable methods, quantifying either microbial biomass (chloroform fumigation extraction [mic_C], total phospholipid fatty acid [PLFA] analysis) or microbial counts (acridine orange direct count [AODC], fluorescence *in situ* hybridisation [FISH] count, quantitative polymerase chain reaction [qPCR]). What must be born in mind is that all of these techniques rely on an extraction of some kind, and oil-derived changes in soil physicochemical properties can reduce extractability (hydrophobic soils repel buffer solutions) as well as cause inhibition in the detection methods (such as PCR inhibition; Paper IV). Lower count or biomass in contaminated soil could thus be partly explained by experimental artefacts, but bias to the opposite direction is improbable.

The general increase in microbial numbers in hydrocarbon-affected soil is attributed to an increase in heterotrophs or specific degrader populations. Most probable number (MPN) enumeration showed an instant growth of fuel oil degraders as a response to hydrocarbon substrate addition (Paper I, fig. 4). As the oil degradation rate decreased, the degrader numbers turned to decrease in the contaminated soils. However, in the uncontaminated reference a consistent upward trend was seen, probably due to increased heterotroph numbers in the legume rhizosphere.

At the aged contaminated site we observed the numbers of degraders of more recalcitrant compounds to respond positively to contamination level (MPN for naphthalene degraders; Wallenius et al., 2011), whereas oil degrader MPN showed no clear trend in the horizontal gradient (Paper III, table 3). Surprisingly, general biomass as mic_C decreased linearly in the three more contaminated plots (Paper III, table 3). This somewhat unexpected result cannot be explained by exceptional TPH content (~0.5 g/kg), since the majority of earlier studies have operated at much higher levels. Repeated application of oily wastes resulting in the accumulation of weathered contamination, as well as chronic exposure, might provide an explanation to the reduction in mic_C at moderate TPH level. On the other hand, soil DNA and RNA concentrations did not respond negatively to aged

oil in the surface soil (Paper IV, table S1), which may reflect the genuine situation or an analytical error. The commercial extraction kit (MOBIO) was different from the one used in the greenhouse experiment (BIO 101), and in preliminary tests DNA yields (but not bacterial community structure) with a kit by the former manufacturer were found somewhat irreproducible.

Besides general microbial numbers and specific degrader populations, microbial ecological studies have also enumerated other properties or groups deemed ecologically important or especially susceptible to contamination (Siciliano & Roy, 1999; MacLeod et al., 2001; Winding et al., 2005). Atlas et al. (1991) predicted an increase in the abundance of plasmid-possessing bacteria in oil-contaminated soil; cultivation-based plasmid enumeration has later been suggested as an efficient pollution indicator (Winding et al., 2005).

Although microbial ecological knowledge has taken major steps forward after the introduction of molecular (culture-independent) methods, important questions concerning the connection between microbial community structure and function are still unanswered (Allison & Martiny, 2008). Thus the enumeration of functional groups does not necessarily provide information on functional rates, which need to be estimated with other methods.

3.1.2 MICROBIOLOGICAL FUNCTIONS

Microbial activity in contaminated soil can be assessed as gross (heterotrophic) activity or with more specialised functions. Whatever the target process, functional microbiological assays in fact always measure potential rates in optimised laboratory conditions with unnatural substrate concentrations; accordingly, the ability of such tests to reflect *in situ* activity has been questioned (Scloter et al., 2003; Winding et al., 2005). In some analyses incubation times of several days enable microbial multiplication (e.g. basal respiration) or even require growth in the incubation medium (substrate usage by Biolog), whereas other tests with a few hours duration typically evaluate the response of the original community (enzyme activity measurements e.g. with ZymProfiler, MicroResp test) (Scloter et al., 2003; Chapman et al., 2007; ISO/TS 22939:2010). Especially for the culture-dependent Biolog tests, preliminary stabilisation and acclimatisation is recommended (Winding et al., 2005), which may affect samples of variable origin very differently.

General heterotrophic activity, depicted by soil respiration, typically increases upon soil contamination due to mineralisation of the added hydrocarbon substrates (Margesin et al., 2003). On the other hand, elevated basal respiration has been interpreted also as a signal of stress or lack of other nutrients relative to carbon (Winding et al., 2005; Bécaert & Deschênes, 2006). Interestingly, light fuel oil pollution was found to have no negative effect on the more specific enzymatic activities measured with ZymProfiler assay either. α- and β-glucosidase, β-xylosidase, cellobiosidase,

chitinase, arylsulphatase, phosphomonoesterase and alanine aminopeptidase behaved similarly in contaminated and uncontaminated soil, whereas leucine aminopeptidase and phosphodiesterase activities were induced by contamination (Paper I, table 3 and unpublished data). Equal to the increase in total microbial biomass, the increased soil enzymatic activities may be explained by higher counts of heterotrophs that use the hydrocarbons as carbon and energy source.

At the landfarming field exposed to repeated crude oil applications, a decrease in basal respiration was observed throughout the horizontal contaminant gradient (Paper III, table 3). Regardless of the depletion in general microbial biomass and activity, enzyme activities were again found relatively insensitive to contamination. Out of the ZymProfiler test kit enzymes listed above, only cellobiosidase and α-glucosidase showed a clear response to oil, correlating negatively with TPH (Spearman rho>0.7 and $p<0.01$ for both; unpublished data).

For some functions, incubation and cultivation bias can be avoided and genuine soil microbial activity detected by measuring processes truly *in situ* with very little sample disturbance: these include at least production of gaseous CO_2, N_2O and CH_4 as well as leaching NO_3^-. Unfortunately, quantifying such fluxes *in situ* is very sensitive to weather conditions, making it often difficult to extract the desired response data from experimental noise (Schloter et al., 2003; Winding et al., 2005). An alternative method for direct functional analysis is qPCR for reverse transcribed messenger RNA (mRNA RT-qPCR), which can provide a snapshot of functional gene expression *in situ* (Smith & Osborn, 2009). This technically challenging method has not yet been successfully applied to the analysis of oil-contaminated sites, but results with other contaminants (Bælum et al., 2008) suggest a promising future if the robustness of the analysis can be improved. That being said, it should be noted that the induction of transcription does not necessarily lead to increased enzymatic activity (Prosser & Nicol, 2008).

3.2 QUALITATIVE COMMUNITY CHARACTERISTICS

Qualitative changes in microbial communities have been studied with a wide variety of community profiling methods that analyse either structural or functional changes. These methods produce semiquantitative data on relative abundances/intensities that is difficult to interpret *per se*, but can be compared within a sample set by visualisation methods and multivariate statistics. The most commonly applied profiling techniques nowadays are PCR-based community fingerprinting methods: denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), automated ribosomal intergenic spacer analysis (ARISA), and length heterogeneity PCR (LH-PCR), the last one utilised in Papers II, III and IV. In structural profiling the target amplified and separated is typically

the gene encoding the small subunit of the ribosome (for prokaryotes the gene encoding 16S ribosomal RNA, from here on referred to as rDNA), whereas functional profiling can target almost any protein coding gene deemed interesting. In both approaches the analysis is in principle exclusive, restricted to those sequences that match the primers used. Different methods have different advantages (see introduction of Paper III), but the choice of a fingerprinting analysis is most often determined by the available apparatus, traditions and personal preferences. Luckily, different techniques usually lead to similar conclusions (Mills et al., 2003; Smalla et al., 2007).

Direct DNA-based molecular methods have many advantages over culture-dependent community analysis, providing usually better coverage of the target community with less labour. Results naturally depend on the precedent DNA extraction, for which multiple manual and commercial protocols exist. Different extraction methods recover DNA of different quantity, purity and diversity, leading to different community analysis results (Delmont et al., 2011b). It is generally assumed that the same extraction method opens the same window to microbial diversity in soil samples with widely differing properties, but this should not be taken for granted (Lombard et al., 2011). The size and quantity of beads used in beadbeating, the decisive step in extraction, can be standardised (ISO/PRF 11063). However, beadbeating efficiency will inevitably differ for soils with different particle size distribution (act as beads too) and organic matter content (buffers the collisions). Likewise, DNA-extraction may be biased in soils with physical properties severely disturbed by oil contamination compared to clean reference soils. In addition, co-extracted humic substances (Paper II) and contaminants (Paper IV) may inhibit PCR. Even though such inhibition is commonly regarded to apply equally to all template sequences, results indicating selective inhibition have been published too (Stach et al., 2001).

3.2.1 DIVERSITY

Diversity is calculated from the number (richness) and relative abundance (evenness) of the more or less dominant operational taxonomic units (OTUs, e.g., separated peaks, bands or sequences) in community data. What must be born in mind is that diversity estimates are not strictly quantitative but qualitative. In comparative microbial community profiling, or even in in-depth explorative community analysis, soil microbial ecologists have no practical way to access the total species richness (Delmont et al., 2011a). Different methods for calculating diversity exist, Shannon and Simpson diversity indices being used the most. The former exaggerates the weight of the rare OTUs and the latter the dominant ones (Hill et al., 2003), but generally different metrics lead to by and large the same conclusions (Gallardo et al., 2011). Even though the diversity figure describes the entire

community, it is a single number on which simple univariate statistics can be conveniently applied (Magurran, 2004).

A rapid decrease in bacterial diversity (i.e., the apparent diversity of the dominant OTUs tracked by LH-PCR analysis) was observed after fuel oil addition in the greenhouse experiment (Paper II, fig. 5). The response mirrored oil-degrader numbers (Paper I, fig. 4) and was reversible, with diversity returning to the same level as the uncontaminated reference upon soil clean-up. Also at the chronically contaminated landfarming field a link between the apparent bacterial diversity and the contamination level was seen (Paper III, table 3). Surprisingly, the average diversity of DNA-based profiles was indifferent in the surface and subsurface soils (Paper IV), reflecting the ability of such methodology to track changes only in the most dominant community members, not the total diversity. On the other hand, clear differences could be observed between DNA and RNA-based profiles of the same sample, the former representing the total community with information on the past included, and the latter representing the active or present community (Girvan et al., 2003; Jansson et al., 2011). In the surface soil, the active community showed greater apparent diversity than the total community (due to higher evenness, but not higher richness), whereas the opposite applied to the more contaminated anaerobic subsurface samples (Paper IV, fig. 1b).

In addition to genetic data, community diversity estimates have also been derived from phenetic properties (i.e., observable characteristics such as cell structure and enzymatic capacity), especially PLFAs. According to Frostegård et al. (2011), such interpretation is misuse of PLFA data, since different fatty acids are not taxonomic units. Clone libraries and second generation sequence datasets, on the other hand, can provide diversity estimates. Although care must be taken in data-analysis (Gihring et al., 2011), these approaches are likely even better suited for diversity analysis than community fingerprinting methods (Bent & Forney, 2008). Their downsides are a bigger workload and higher cost per sample, which also applies to the analysis of community DNA by reassociation kinetics. This last technique is a conceptually superior method for truly quantifying the genetic diversity of soil microbial communities, and it has revealed a decrease in diversity due to herbicide and heavy metal pollution (Atlas et al., 1991; Sandaa et al., 1999). Unfortunately, no results on hydrocarbon polluted soils exist, and the use of this technically demanding method seems to be restricted to a few specialised laboratories.

Although diversity assessment is a practical way to simplify and compare community profiles or even sequence datasets, structural analysis of community data with multivariate methods is more likely to reveal ecologically significant changes (Hill et al., 2003; Hartmann & Widmer, 2006).

3.2.2 COMMUNITY STRUCTURE

The comparative analysis of microbial community structure is what is usually described in the majority of studies that claim to investigate microbial diversity. The input data is similar to diversity calculation – presence/absence or relative abundance of different OTUs – but in this approach the OTUs are typically also classified (binned) to categories, which must be congruent in the entire sample set. Structural assessment is by definition comparative and requires the analysis of several differing samples to be sensible, analogical to the analysis of β-diversity (dissimilarity of spatially or temporally distinct communities; Anderson et al., 2011).

The original way to compare community compositions was to present the fingerprint raw data – due to limited figure space in publications representative or averaged profiles can also be shown (Paper II, fig. 4). However, mere visual assessment provides a limited view of the data, which can be converted from figures to numbers and analysed with a wide variety of clustering and ordination methods (Ramette, 2007). These require a number of justified decisions on how to analyse the data, the first and perhaps most important one concerning the OTU input format: binary or semiquantitative, shared absences excluded or included. Binary analysis is by definition sensitive to the detection threshold. Relative abundances, on the other hand, can be quantified based on either peak height or area (Culman et al., 2008). Data analysis approaches that do not regard joint OTU absences to increase community similarity are generally favoured in ecology, but the contrary strategy can also be justified, especially in the study of environmental disturbance (Anderson et al., 2011). Correct binning of the OTUs is especially crucial, and if the dataset is too large to check and adjust the bins manually, advanced algorithms are required to ensure this (Ramette, 2009). Very different conclusions have been drawn with binary and proportional input data (Lozupone et al., 2007); however, after these primary analysis decisions, similar methods typically give similar results (Anderson et al., 2011). One way to simplify data analysis is to not detect, quantify and classify distinct OTUs (peak-based analysis), but instead analyse the intensity of the aligned fingerprints on the whole data range (curve-based analysis). Paper II demonstrated that the latter approach is applicable to all multivariate community analysis approaches commonly used in ecological studies: unconstrained and constrained ordination, hypothesis testing, and identification of species responsible for the major observed community changes (Anderson & Willis, 2003).

Community structure has been commonly reported to respond sensitively to hydrocarbon contamination – fewer reports exist on its suitability for monitoring community recovery upon soil remediation. Bacterial community reacted dramatically to a single light fuel oil addition; the response was distinct one week after contamination, and though the size of the effect diminished upon the biodegradation process, the difference to the uncontaminated reference remained significant even when the soil was

legislatively clean (Paper II, fig. 1). The difference in community structure could be attributed to an increase in the peak of size 521 bp, the relative abundance of which correlated with TPH dissipation rate (Paper II, fig. 4). Interestingly, unconstrained ordination of all the 180 LH-PCR profiles from the greenhouse experiment showed that community recovery upon soil clean-up was not explained by the community returning to its original state but by the development in the same direction as the clean control soil (Paper II, fig. 2). Obviously, without appropriate references no conclusion on the bacterial community recovery could have been made.

At the field site with aged crude oil pollution, constrained ordination with non-parametric canonical correlation analysis (CCA) revealed that the contamination level shaped bacterial community structures. The decisive role of the oil concentration was detected with the total dataset (Paper IV, fig. 2A) as well as surface soils only (Paper IV, fig. 2B). Intriguingly, at the surface the RNA-based "present community" fingerprints resembled the DNA-based "past+present community" fingerprints in samples with a >1 g/kg lower TPH level (Paper IV, fig. 2B), possibly signalling that the soil was already recovering from the perturbation. No such trend or dependence on contamination level as the major determinant of community structure could be observed with the subsurface dataset (Paper IV, fig. 2C).

In both experiments, the variation explained by unconstrained ordination (principal coordinate analysis) and eigenvalues for the axes of constrained ordination (CCA) were exceptionally high (Papers II & IV; Cottenie, 2005). The curve-based fingerprint data analysis approach (providing possibly a more "full" picture of the total bacterial community due to minimal data binning) and rather similar samples probably contributed to this phenomenon. However, this result may also reflect the critical effect of oil contamination on microbial communities and other soil variables.

Besides 16S rDNA-based techniques, functional profiling with various catabolic genes is applicable for the analysis of degradative community development. Also phenetic profiling with PLFA, as well as community level physiological profiling (CLPP) with Biolog and MicroResp, have been found suitable for community comparisons (MacLeod et al., 2001; Chapman et al., 2007; Frostegård et al., 2011). The problem with these phenetic methods is that their results are usually a dead end, revealing effects but little chance to investigate what contributes to the observed changes. Genetic comparisons, on the other hand, can be complemented with sequence analysis that provides taxonomic information on the changing community members.

3.2.3 COMMUNITY COMPOSITION

Even though novel techniques based on spectral or protein analysis are being developed, knowledge on microbial community composition is currently predominantly derived from sequence data (either Sanger or second generation sequencing). Reference databases are growing at an exponential

rate, and regardless of the numerous prokaryotic phyla with no cultured representatives, the coverage is becoming reasonable for at least some ecological inference especially with 16S rDNA (SILVA rRNA database project; Pruesse et al., 2007). The part of the gene with best taxonomic separation power is the first third containing hypervariable regions V1-V3 (Jeraldo et al., 2011); conveniently, the same region is amplified and analysed in many fingerprinting protocols, including LH-PCR used in Papers II, III & IV. In methods utilising gel separation like DGGE, distinct bands can be cut, amplified and sequenced directly. However, microbial diversity even in severely contaminated soils is typically so high that products amplified with general bacterial primers usually require cloning for a good quality sequence. The production of clone libraries or second generation sequence datasets (the latter typically with the 454 or Illumina platforms; Glenn, 2011) involves random limited sampling, more steps and more bias than community fingerprinting (Wintzingerode et al., 1997; Zhou et al., 2011b). Even though especially the latter technique produces superior amounts of sequence data on community composition, fingerprinting methods may still be better for rapid and reproducible assessment of structural community changes.

Because the majority of the genera known to be capable of hydrocarbon utilisation (Prince et al., 2010) belong to taxa that are common in soil (Janssen, 2006), mere taxonomy seldom provides sufficient evidence for effective oil degradation. However, an increase in abundance due to hydrocarbon addition is usually regarded as a sign of a degrader. Such a response was seen with *Aquabacterium* in the fuel-oil polluted greenhouse soil – this taxon with amplicon length 521 bp in LH-PCR was detected exclusively in the contaminated soils, and only during the steep TPH dissipation (Paper II). Strong evidence on betaproteobacterial *Aquabacterium* consuming alkanes, the dominant fraction of fuel oil, is surprising – Gammaproteobacteria usually respond more rapidly to utilise these easily biodegradable compounds (Militon et al., 2010). Interestingly, many readily culturable soil *Pseudomonas* isolates that are able to use fuel oil as the sole carbon source were found to produce the same amplicon length 521 bp with different sequence (Wallenius et al., 2011; unpublished data from both experiments). Thus the identification of degraders with LH-PCR screening of isolates, instead of LH-PCR screening of clone libraries, would likely have resulted in erroneous conclusions.

At the landfarming field, analysis of both DNA and RNA based community fingerprints and clone libraries enabled inference of the activity of the identified taxa. Many of the identified bacteria in the surface soil were classified into groups with known hydrocarbon degraders (Prince et al., 2010), such as Burkholderiales, Actinomycetales including *Mycobacterium*, as well as Xanthomonadaceae. These taxa were metabolically active (relative abundance in RT-LH-PCR profiles comparable to abundance in LH-PCR profiles; Paper IV, fig. 5), implying that substrates for hydrocarbon

degraders may still be available regardless of the heavily weathered nature of the crude oil. However, *Sphingomonas*, known for its capacity to degrade recalcitrant organic contaminants (Kertesz & Kawasaki, 2010), was relatively inactive. In addition to these aerobic degraders, some of the identified dominant taxa have been connected to anaerobic alkane degradation, namely Chloroflexi and *Geobacter* (Mbadinga et al., 2011). This observation indicates that anoxic conditions may limit biodegradation even in the regularly tilled top 20 cm of soil, possibly due to soil physical properties being degraded by oil contamination (Paper III). On the other hand, anaerobic niches can be found even in generally aerated soil (Lombard et al., 2011), and also good aggregate formation and strong structure of the soil have been claimed to increase anoxic microniches in surface soils (Ollivier et al., 2011).

Besides taxa presumed to be capable of utilising oily substrates, oxidisers of nitrate (Nitrosomonadales) and sulphur (Acidithiobacillales) were detected, along with Acidobacteria (Paper IV, fig. 5). The lastly mentioned phylum is common especially in undisturbed soils (Janssen, 2006) and has been reported to react negatively to oil pollution (Saul et al., 2005). However, our results suggest that this taxon, even though observed to respond to an increase in the contamination level by a community composition change (from Group 6 to Group 1), is not by default sensitive to crude oil contamination.

Although aerobic hydrocarbon degradation pathways are relatively well characterised, much less is known about anaerobic oil degradation. In addition, the anoxic catabolism of hydrocarbons generally produces so little energy and takes place so slowly that studying these processes is not straightforward (Mbadinga et al., 2011). Interestingly, in the dense and anaerobic subsurface layers with only slightly weathered oil (Paper III), we found active populations of bacteria associated to anaerobic alkane degradation: Desulfobacterales, Chloroflexi and *Smithella* (Paper IV, fig. 5; Mbadinga et al. 2011; Gray et al., 2011). Bacteroidetes were abundant in DNA but relatively fewer in RNA-based profiles, suggesting that they might have originated also from the oily waste water treatment plant instead of being authentic members of the active oil-degrader community – however, also this phylum has been detected in anaerobic oil-contaminated environments (Siddique et al., 2011). Drawn together, the dominance of degradative taxa suggests that hydrocarbons are the predominant substrates in the compacted and heavily polluted subsurface soil, and the potential for crude oil biodegradation exists even in such an extreme environment.

Only very few reports on archaeal community composition in oil-contaminated unsaturated soil have been published. Changes in community structure at the landfarming site were investigated with LH-PCR targeting archaea, but the profiles with typically two peaks were so simple that multivariate analysis was unnecessary (Paper IV, fig. 3). Clone library construction and sequencing revealed that archaeal communities were very simple, meaning that the simplicity of the LH-PCR profiles was not a flaw of

the method but a real result. In the surface soils only Thaumarchaeota were detected, and even these solely in the less contaminated half of the horizontal gradient – no archaeal sequences could be amplified from the plots with a TPH level >4 g/kg. Group I.1b (*Nitrososphaera*-like sequences) seemed more active than group I.1a (*Nitrosoarchaeum*-like sequences) (Paper IV, fig. 4).

Unexpectedly, the amplification of archaeal sequences for LH-PCR and clone library construction was more successful from the heavily polluted subsurface soils, possibly reflecting a higher proportion of archaea compared to bacteria in these extreme conditions (Paper IV, fig. 3). Sequences represented two active lineages that have been detected also before in anaerobic environments, but have no cultivated representatives. The Miscellaneous Crenarchaeotic Group (MCG, i.e., group I.3b; Paper IV, fig. 4) are likely anaerobic heterotrophs that assimilate complex organic substrates (Teske & Sørensen, 2008), but have not been earlier associated specifically to hydrocarbon-pollution. A homogenic group of sequences belonging to the Arc I cluster was also identified (Paper IV, fig. 4). This clade recently suggested by Chouari et al. (2005) has also been found in contaminated soils (Sekiguchi, 2006) and probably consists of hydrogenotrophic or acetoclastic methanogens (Rivière et al., 2009). Interestingly, we detected no sequences of known methanogenic orders commonly found in oil-impacted environments, even though syntrophic methanogens are required to consume hydrogen for alkane oxidation in methanogenic conditions (in the absence of sulphate) to be thermodynamically viable (Gray et al., 2011). It is likely that known methanogens remained undetected due to primer bias, or sulphate was still present in the dense severely contaminated subsurface and rendered the redox potential unfavourable for methanogenic processes. It may also be speculated whether the Arc I group archaea could act as the methanogenic partners in syntrophic methanogenic alkane degradation in the studied anaerobic soil.

Typically the focus in community composition analysis is on one or a few marker or functional genes. An exception to this is the whole-genome metagenomics approach, which seems very promising also for environmental change studies (Zhou et al., 2011a). However, the cost of the analysis (including data analysis) renders it still unpractical for the majority of comparative environmental studies. The routine application of micro-arrays, e.g., Phylochips or Geochips, could be much nearer in the future. In the array methods the community composition data is not traced by sequencing but through specific hybridisation to a wide set of selected probe sequences. Arrays have already been successfully applied to the study of hydrocarbon-contaminated soils (Liang et al., 2009; Van Nostrand et al., 2010), and especially promising is the possibility for direct RNA analysis without amplification bias (DeAngelis et al., 2011).

Table 2 *Summary of the responses of soil microbial ecological variables to hydrocarbon contamination (temporal response to pollution event or spatial response to increasing pollutant concentration).*

		Paper I	**Paper II**	**Paper III**	**Paper IV**
Case	Contaminant	Freshly added fuel oil (TPH 3 g/kg)		Weathered crude oil (TPH surf. 3-7, subsurf. 5-46 g/kg)	
	Location/ study design	Destructive greenhouse experiment		Analysis of *in situ* gradients at oil refinery waste landfarming field	
Microbiological soil analyses	Quantitative analyses	Increase in microbial biomass, reversible increase in fuel oil degraders, no effect or increase in enzyme activities	-	Decrease in heterotroph activity, decrease in microbial biomass, no effect on fuel oil degraders (increase in naphthalene degraders, no effect or decrease in enzyme activities)	Decrease in microbial biomass and activity
	Qualitative community analyses	-	Reversible decrease in apparent bacterial diversity, largely reversible effect on bacterial community structure, reversible dominance of *Aquabacterium*	Decrease in apparent bacterial diversity, effect on bacterial community structure	Effect on bacterial community structure in the surface: same direction but different effect level for present and historical community, negative effect on Thaumarchaeota

4 MICROBIOLOGICAL INDICATORS OF SOIL QUALITY

Soil quality can be regarded good if the soil can function to deliver those services that we expect from it (Karlen et al., 2003; Haygarth & Ritz, 2009). The necessary functions are generally connected to soil structure and biogeochemical cycling of elements, the maintaining of which is governed by the activity of micro-organisms. Accordingly, there is nowadays a broad agreement that microbiological tests should be included in the evaluation of soil quality (Winding et al., 2005; Ritz et al., 2009). Microbial variables can respond to environmental pressures more rapidly than soil physicochemical characteristics, being thus valuable early indicators of change (Schloter et al. 2003; Winding et al., 2005; Bécaert & Deschênes, 2006). Investigating the changes in microbiological functions is deemed especially relevant, but unfortunately these are difficult to monitor, as explained under section 3.1.2. Moreover, the attempts to open "the black box of soil microbial activity" – linking community structure to metabolic potential, to realised functions, to environmental fluxes – are still hindered by both methodological (Jansson et al., 2011) as well as theoretical (Prosser et al., 2007) hurdles. However, alteration of microbial community structure almost necessarily has an impact on its functional properties in the long term (Allison & Martiny, 2008). According to Allison & Martiny (2008), it would therefore be short-sighted to neglect the structural properties of microbial communities when modelling environmental change and ecosystem services.

In traditional soil science, the concept of soil quality usually refers to the proper or inadequate agronomical functioning of soil (Karlen et al., 2003; Bécaert & Deschênes, 2006). Soil is of sufficient quality if it is able to sustain the agricultural and forestry production of renewable goods: food, fodder, fuel and fibre. A related term with a somewhat different ring is soil health. This concept is more intuitively associated with the ecological status of the soil, its ability to provide non-agricultural ecosystem services sustainably in the long run (Bécaert & Deschênes, 2006). Parallel and even synonymous use of soil quality and health seems justified from the point of view that ecologically healthy soil is required also for the sustainable production of renewable goods, even more so under increasing demands and decreasing inputs (Kibblewhite et al., 2008). Moreover, certain elementary processes, related to the maintenance of soil structure as well as the cycling of elements and water, are required even at severely disturbed industrial areas – and additional ones if the site is required to support plant growth (Winding et al., 2005). Thus, the assessment of soil quality or health should not be restricted to land used for food production.

An indicator is a qualitative or quantitative property that can tell more about the status of the studied system than just its own measured value

(Niemeijer & de Groot, 2008); body temperature serves as such an indicator for human health, whereas for soil health, for example, the ratio of clay to organic carbon has been suggested (de Jonge et al., 2009). Due to their dynamic and central role in many soil ecosystem processes, the value of microorganisms as environmental indicators has been recognised (Schloter et al. 2003; Winding et al., 2005; Kibblewhite et al., 2008). However, discussion on which soil biological properties would best characterise soil quality is still active (Ritz et al., 2009). Indicators in general should be SMART: specific, measurable, achievable, relevant and time-bound (Niemeijer & de Groot, 2008). The advantage of prokaryotic micro-organisms over eukaryotes is their universal abundance – especially bacteria can be detected in any soil or soil-like environment. Each indicator should also have a significant role in the indicator set (Niemeijer & de Groot, 2008): there should be high uncertainty about the value of the indicator, which should not be directly derivable from other (more readily measurable) properties. With some microbiological variables this may be problematic, as their high correlation with, e.g., soil organic carbon content – interestingly highlighted as an advantage in most microbiological publications – is actually a good reason to leave such a redundant indicator unmeasured.

Because of the heterogeneity of natural environments and biological processes – contaminated soil as an extreme example – a large number of replicates are usually required for statistically sound studies. Reproducible, analytically easy and cost-efficient methods should better help cope with the high variability. An even bigger challenge in environmental microbiological studies may be measurement uncertainty, because these microscopic organisms and their functions cannot be observed directly. Due to practically unavoidable bias associated with storage especially in degradation or growth-allowing conditions (Wallenius et al., 2010; Gonzalez-Quiñones et al., 2011), pre-treatment and cultivation, methods able to avoid these steps seem generally more justifiable.

Soil quality should be evaluated from the point of view of expectations, determined by the intended land use (Karlen et al., 2003; Haygarth & Ritz, 2009). However, different disturbances may risk different functions and ecosystem services. In addition, the response of environmental indicators may vary depending on not just the extent but also the nature of the impact. Consequently, it may be necessary to adjust the set of soil quality indicators and/or their interpretation also depending on the expected (anthropogenic) threat.

4.1 SOIL MICROBIAL INDICATORS FOR DELETERIOUS ECOLOGICAL EFFECTS OF HYDROCARBONS

During the past 10-20 years, there has been a growing interest in microbiological analyses indicating negative impacts of hydrocarbon

contaminants on soil functioning (Brassington et al., 2007). MacLeod et al. (2001) stressed that there is a need for *in vivo* assessment of microbiological bioavailability of contaminants, but did not see one available in the near future, due to the vast complexity of hydrocarbon compounds and soil as an environment. They concluded:

> *"The development of microbial tests that measure the assessment endpoints for a wide range of contaminants in various soils and exposure conditions is one of the largest challenges for microbial ecologists."*

Paton et al. (2005) pointed out that the microbiological tests currently used to evaluate soil quality differ for agricultural and contaminated soils. Bécaert & Deschênes (2006) and Schloter et al. (2003) emphasised that an indicator should work equally well in all environments. What seems evident is that methods depending on microbial growth and/or aerobic respiratory activity under incubation (such as cultivation techniques including Biolog, *V. fischeri* photobacterium test, and basal respiration) are more easily biased and not readily applicable to anaerobic soils, which are rather common in oil-polluted sites, including the landfarming field studied in Papers III & IV. The selection of indicators is a crucial step in the evaluation of polluted soil quality also because not all contaminants – even all hydrocarbon contaminants – have the same effect (Bécaert & Deschênes, 2006).

According to Winding et al. (2005), soil respiration, organic matter degradation and microbial biomass are good classical microbial ecological methods that should be included in the assessment of soil quality. Also Bécaert & Deschênes (2006) and Schloter et al. (2003) recommended microbial biomass and activity. Paton et al. (2005) listed these same methods as microbial parameters for soil ecotoxicity testing, but admitted that there are inconsistencies between the assays. Contrastingly, Semple et al. (2003) claimed that the traditional techniques for monitoring soil microbes are insensitive to hydrophobic organic pollutants, and instead mirror the effect of incubation and other artefacts.

What is evident is that *in situ* soil microbial ecological indicators do not respond exclusively to oil pollution but are an integrated measure for the changed conditions: deteriorated soil physicochemical properties and reduced primary production. In Paper III, the drop in soil pH probably contributed to the decrease in general microbial activity in the horizontal contaminant gradient, especially because fungi relatively more active in acidic soils (Rousk et al., 2010) were not competitive due to the biannual tillage. Plants were found relatively sensitive to both fresh and aged oil pollution (Papers I & III), and toxicity to vegetation can affect both rhizosphere inhabitants as well as the entire carbon cycling in soil. For example, reduced plant biomass in the contaminant gradient was quite possibly the decisive factor for the decrease in the activities of cellobiosidase and α-glucosidase, enzymes that degrade cellulose and starch (unpublished

data). Passive *in situ* bioassays reflect also the collective stress from competition and predation (Paton et al., 2005; Fränzle, 2006). Predation and viruses typically "kill the winner" (Fuhrman, 2009), but predation may be relatively less significant in disturbed environments (Torsvik et al., 1996). A decrease in microbial community evenness due to oil pollution may thus be a combined effect of hydrocarbons favouring the degraders and harming the predators. Indeed, the generally higher sensitivity of higher organisms was suggested by the plant results (Paper I). However, the numbers of nematodes (mostly herbivores) on a slightly less contaminated sector of the landfarming fields were found to be on the normal range (A. Pulkkinen, unpublished data).

In general, microbial ecological indicators related to carbon cycling could be useful due to the principal importance of organic matter, as a reserve of nutrients and general indicator of soil quality (de Jonge et al., 2009). Specifically, Winding et al. (2005) note heterotrophs as a good indicator class due to their abundance and role as the agents cycling all organic carbon. In addition, microbial modification of organic matter is crucial for the physical soil attributes – structure and hydrological properties – which largely determine how the soil functions (Young & Crawford, 2005; Or et al., 2007; Kibblewhite et al., 2008; Abiven et al., 2009).

Microbial biomass, reflecting the gross sum of the dominant heterotrophs and the generally less abundant autotrophs in soil, is consistently included in lists of recommended microbial soil quality indicators (Schloter et al., 2003; Winding et al., 2005; Bécaert & Deschênes, 2006; Ritz et al., 2009; Gonzalez-Quiñones et al., 2011). Although Schloter et al. (2003) admit that different biomass in different soils does not necessary mean a difference in soil quality, none of the earlier works acknowledge the fact that microbial biomass and/or numbers typically increase due to oil-pollution (Paper I; Chaîneau et al. 1995; Joergensen et al. 1995; MacLeod et al., 2001; Margesin et al., 2007; Bundy et al., 2004; Coulon et al. 2005; Marin et al. 2005; Rončević et al. 2005; Serrano et al. 2008; Kostka et al., 2011). The original publications relate this result to hydrocarbons acting as growth-supporting assimilable substrates. The review articles on microbial ecological indicators for soil contamination mention only other contaminants but not crude or refined oils (MacLeod et al., 2001; Gonzalez-Quiñones et al., 2011). Kefford et al. (2008) worried that considering also hormesis, i.e., the seemingly beneficial effect of the toxic contaminant at low concentrations, as a toxicological response may “open Pandora’s box” in the field of ecotoxicology. However, an increase in microbial numbers due to oil addition is clearly not insensitivity but an effect, which cannot be neglected or explained by low contamination level: even in Paper I, with one of the lowest TPH concentrations, the negative effect on plant growth was evident. Interestingly, in Paper III the weathered crude oil contamination was observed to chronically decrease microbial biomass, regardless of more abundant substrates. The explanation to these deleterious effects at rather

low TPH level could lie in the contaminants not recovered by TPH analysis as well as the repetitive application of the pollutants during the 25 years of landfarming activity. According to Rončević et al. (2005), soil can recover from a single pollution event, but the effects of repeated soil hydrocarbon contamination are detrimental.

Whereas microbial biomass reflects heterotroph numbers, soil basal respiration is a proxy of their activity. Also this indicator typically responds positively to oil contamination (Rhykerd et al., 1995; Joergensen et al., 1995; Bundy et al., 2001; Marin et al,. 2005). Although mineralisation of the hydrocarbons is the obvious explanation, elevated respiration has also been inferred as a stress response (Joergensen et al., 1995; Franco et al., 2004). At the landfarm site, the basal respiration rate decreased throughout the horizontal contamination gradient *in situ*, being the most sensitive microbial indicator (Paper III). Besides chronic and repeated contamination, also the quality of the pollutant may explain this result: Bundy et al. (2001) found diesel and three lighter crude oils to induce respiration, whereas the heaviest crude oil inhibited it. They expected the lighter or refined oils to exert higher toxicity, but this was obviously not the case – on the other hand, their results could be explained by more ready mineralisation of the lighter hydrocarbons, and the heavy crude impairing soil aeration. For our *in situ* results, only the latter explanation stands – together with the assumption of higher toxicity at higher contamination level – since in the more polluted plots also the concentration of the readily degradable alkanes was higher (Paper III). In contrast to basal respiration, enzyme activities, another measure of heterotrophic activity, were found relatively insensitive to both fresh (Paper I) and aged hydrocarbon contamination (unpublished data).

After carbon, nitrogen is the second most abundant element in dry biomass (Madigan et al. 2003) and often the nutrient restricting plant growth – and hydrocarbon biodegradation. The processes of nitrogen cycling have been suggested as indicators of soil quality and pollution; especially biological nitrogen fixation and mineralisation of organic nitrogen are regarded relevant since they reduce dependence on fertilisers (Schloter et al., 2003; Winding et al., 2005; Ollivier et al., 2011). Although light fuel oil contamination retarded the growth of fodder galega, it did not compromise symbiotic nitrogen fixation by *Rhizobium galegae* (Paper I); in fact, biological nitrogen fixation by legumes and their rhizobial symbionts has even been suggested to be induced by oil contamination (Carr, 1919). For routine environmental monitoring legume-symbiont experiments are rather laborious and depend on the overall sensitivity of the plant to the pollutants. However, the *nifH* gene could serve as a general indicator of nitrogen fixation (Ollivier et al., 2011). Direct molecular analysis of nitrogen mineralisation is unfortunately hampered by the multitude of related taxa and pathways (Winding et al., 2005). On the other hand, enzymatic assays, such as aminopeptidase activity, are related to nitrogen mineralisation (Schloter et al., 2003). In Paper I,alanine aminopeptidase was unaffected by fuel oil

whereas leucine aminopeptidase activity was induced, possibly due to he relative shortage of nitrogen to carbon; in the crude oil-contaminated site no clear trends were observed for either (unpublished data). In conclusion, soil quality indicators related to nitrogen fixation and mineralisation do not seem readily applicable for routine monitoring of the effects of hydrocarbons on soil health.

As reviewed above, the responses of heterotrophs to hydrocarbons are inconsistent – thus autotrophic bacteria and archaea that cannot use oil as carbon and energy sources could be more useful indicators of the detrimental impacts of pollution. Indeed, community profiling (Ritz et al., 2009) and quantification (Wessén & Hallin, 2011) of autotrophic ammonia oxidising bacteria and archaea have been suggested as general soil quality indicators. Thaumarchaeota, putative ammonia oxidisers globally abundant in soils (Bates et al., 2011), indeed seemed to respond negatively to aged oil contamination, as they could be detected only in the less polluted half of the horizontal contaminant gradient (Paper IV). Archaea in general have in water-saturated environments been reported to react on oil-pollution more sensitively than bacteria (Röling et al., 2004), and there are no known mesophilic hydrocarbonoclastic (hydrocarbon-degrading) archaea (Prince et al., 2010). Contrastingly, Ollivier et al. (2010) claimed that archaea may be generally more resistant against xenobiotics than bacteria, which is well in accordance with their more robust cytoplasmic membrane composition (Valentine, 2007). In addition, ammonia oxidising bacteria and archaea in general could be favoured by nitrogen fertilisation and a lack of plants competing for ammonia (Ollivier et al., 2010), so they might even thrive in contaminated soil biostimulated with fertilisers. Kurola et al. (2005) monitored ammonia oxidising bacteria, generally regarded sensitive to soil pollution, at the aged contaminated site also studied in Papers III & IV. They found no negative impacts but an adapted, stable and active ammonia oxidiser community. Mußmann et al. (2011), studying nitrifying sludges from waste water treatment plants, recently questioned the generally assumed ecophysiological role of ammonia monooxygenase gene containing archaea as autotrophic ammonia oxidisers. Interestingly, their results suggested that Thaumarchaeota (group I.1b) were in fact favoured by petroleum refinery wastes and might use these heterotrophically.

Fungi are another group of micro-organisms that according to current knowledge cannot benefit from hydrocarbons by utilising them as a sole source of carbon and energy (Valentín Carrera, 2010). Especially mycorrhizal fungi have been suggested as relevant and sensitive soil quality indicators (Schloter et al., 2003; Winding et al., 2005). However, contrary reports showing insensitivity of mycorrhizal fungi to oil pollution also exist (Sarand et al., 1998). Traditional methods based on cultivation with the plant host are labour-intensive, but molecular methods are promising also for the direct culture-independent study of fungi. Also *in situ* the abundance of mycorrhiza is dependent of the growth of the host plant, and thus typically requires non-

tilled aerobic soil. On the other hand, trapping of mycorrhizal fungi from unvegetated soil samples with trap plants does not necessarily accurately reflect the restrictions of mycorrhizal symbiosis *in situ*. At the landfarming field with weathered oil pollution, no mycorrhizal structures could be detected in the roots of the native weeds. However, mycorrhiza – *Glomus intraradices* and *G. fasciculatum* as the most abundant species – could be successfully trapped from the soil with *Plantago lanceolata* and *Phalaris arundinacea* seedlings (M. Vestberg, unpublished data), suggesting that at least spores of mycorrhizal fungi endured the heavy hydrocarbon contamination.

The degraders of hydrophobic contaminants are commonly quantified upon microbiological assessment of contaminated soil. According to Semple et al. (2003), these organisms are essential to soil health and fertility in general. When enumerating them from soil under restoration, increased numbers are typically a positive signal of biodegradation activity (Paper I). However, in the evaluation of contaminated soil quality, high degrader numbers must mean that there is plentiful accessible food for this group to give them a relative competitive advantage. As the bioavailability of hydrocarbon contaminants is the principal determinant for both degradation and toxicity, availability to degraders may also indicate availability to more sensitive soil organisms (Semple et al., 2003; Grotenhuis & Rijnaarts, 2011). However, the organisms specialised in utilising hydrocarbons have improved strategies to enhance access to and mass transfer of these poorly soluble compounds (Van Hamme et al., 2003; Stroud et al., 2007; McAllister & Semple, 2010). The logical outcome is that if oil is not bioavailable to be degraded, it is highly unlikely to exert toxic pressure either. Thus the relevant hydrocarbonoclastic groups – degraders of fuel oil in the greenhouse experiment (Paper I) and degraders of the more persistent naphthalene at the landfarming field (Wallenius et al., 2011) - seem suitable *in situ* indicators of bioavailability also in ecological risk assessment. In contrast to general heterotroph analyses, bioavailability estimation through degrader enumeration actually benefits from the ability of hydrocarbons to exert strong selective (favouring) pressure as a rich source of carbon and energy. However, degrader numbers alone bear little information on the soil microbial ecological condition if studied in isolation, as their response may be either similar (Paper I) or contrary (Wallenius et al., 2011) to the general response.

Plasmids often confer degradation capacity and resistance, and they have been suggested to increase in abundance in oil-contaminated soil (Atlas et al., 1991). Hence plasmid prevalence in the native bacterial community could be used as a proxy of soil contamination (Winding et al., 2005). If more studies confirm this assumption, and analysis methods less dependent on the culturability of soil bacteria and/or their conjugability with test strains can be developed, plasmid enumeration from indigenous soil bacterial communities could be a highly useful general indicator of soil pollution.

Compared to the majority of other methods, the advantage of this indicator is to collectively reflect stress from mixed contamination, both organic pollutants as well as heavy metals. Pollution-induced community tolerance (PICT), on the other hand, is a contaminant-specific single end point method that should specifically reduce the risk of false positives in the assessment of pollution effects (Siciliano et al., 2000; Winding et al., 2005). This method evaluates the adaptation of the microbial community by measuring functional resistance to increasing concentrations of the pollutant, typically through Biolog analysis. As a result, the method only quantifies the resistance of the culturable bacteria, and it has so far not been tested with crude oil or oil products.

As explained above, careful consideration is needed in the evaluation of polluted soil quality through quantitative microbial measures: which changes actually are positive and which negative (Winding et al., 2005). Typically, increase in microbial numbers and activities is intuitively regarded positive (Moreno et al., 2011), even though MacLeod et al. (2001) point out that an adverse response can be either an increase or a decrease. Interestingly, many of the commonly suggested soil microbial ecological indicators are also intrinsically contradictory. For example, soil respiration generally means good microbial activity and healthy C cycling – however, it can also result in depletion of soil organic matter. Ammonia oxidation indicates undisturbed N cycling, but leads to N losses through nitrite leaching and denitrification (Ollivier et al., 2011). Substrate-induced respiration (SIR) has been traditionally used to quantify soil microbial biomass, but r-strategic bacteria are by definition better at rapidly utilising the readily available added substrates, and are favoured over K-strategists under unstable disturbed conditions (Margesin et al., 2003; Fierer et al., 2007).

If direct interpretation of changes in microbiological numbers or activities is a dead end especially in the assessment of hydrocarbon contamination effects, what alternatives exist? Sun ray or amoeba presentations, recommended by Schloter et al. (2003) and Moreno et al. (2011), ease simultaneous visualisation of several quantitative indicators; unfortunately, they do not solve the original problem, especially in the case of the commonly recommended but contradictory biomass and respiration. The sum or product of different indicator values will do no better. Winding et al. (2005) and Bécaert & Deschênes (2006) called for reference values, but because of the multitude of factors affecting microbial measures, successful establishment of specific reference levels for each situation is quite unlikely. Altogether, single counts or rates generally seem of little use without knowledge on their relative location on the response curve (indicator value as a function of contamination level and/or time after contamination). Moreover, such response curves are not necessarily linear but can be also unimodal or asymptotic. What is evident is that changes in microbial indicators must always be interpreted with relation to changes in

physicochemical variables (Schloter et al., 2003; Bécaert & Deschênes, 2006).

Quotients, ratios of two variables, could be more useful than individual indicators (Schloter et al., 2003). A commonly used ratio is the metabolic quotient qCO_2, a ratio of basal respiration and microbial biomass, the latter quantified through substrate-induced respiration. Although this quotient should indicate changes in the metabolically active proportion of soil microbes and has been used as a measure of general stress, it has also been reported to infer ecosystem immaturity or substrate addition (Wardle & Ghani, 1995; Winding et al., 2005). Changes in applied metabolic quotient (gross microbial activity / microbial biomass) were calculated from data in Papers I & III. Results presented in Figure 6 show that this quotient does not seem directly applicable for the evaluation of deleterious effects of hydrocarbons on soil microbial ecology.

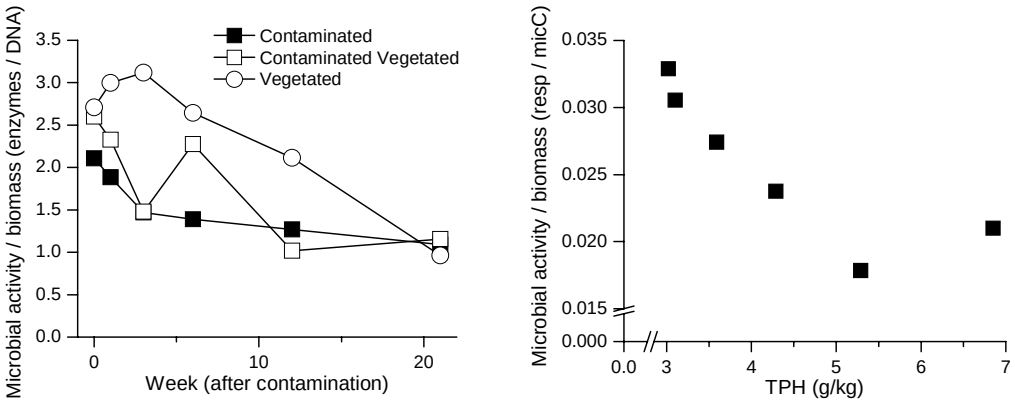

Figure 6 Response of applied metabolic quotient to hydrocarbon contamination. On the left, temporal development after a single fuel oil contamination to 3 g/kg (sum of the ten measured soil enzyme activities [µmol/(3 h × g soil)] / soil DNA[µg/g soil]); On the right, spatial response to contamination level difference *in situ* (basal respiration [CO_2 mg/(h × kg soil)] / microbial biomass carbon [mg/kg soil]).

The effect of oil pollution on heterotrophs is variable, but hydrocarbon degrader numbers will usually respond positively. Thus the ratio of degraders to total biomass should infer hydrocarbon bioavailability. Results calculated from Papers I & III and Wallenius et al. (2011) show that the response of degraders/total quotient is predictable in both hydrocarbon-contaminated soils with contrasting properties (Figure 7). What should be noted is that the method used to quantify degraders – MPN, plate count or qPCR – should not make a difference, if the degrader group is relevant to the contamination and the technique is reproducible and consistently applied on the whole sample set.

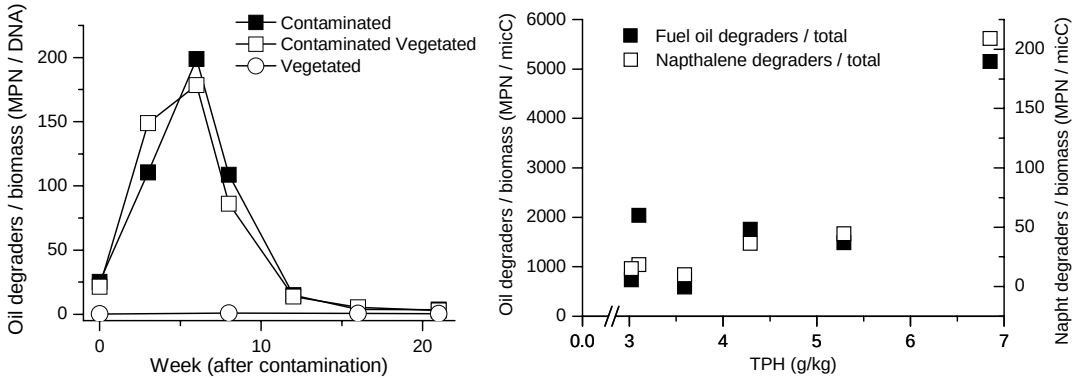

Figure 7 Response of the hydrocarbon degraders / total microbial biomass ratio to hydrocarbon contamination. On the left, temporal development after a single fuel oil contamination to 3 g/kg (fuel oil degrader MPN [MPN/g soil]/ soil DNA [µg/g soil]); On the right, spatial response to contamination level difference *in situ* (oil and naphthalene degrader MPN [MPN/g soil] / microbial biomass carbon [mg/kg soil] – two degrader groups quantified one year apart).

Qualitative microbial community analysis is commonly recommended to be included in soil quality estimation (Scloter et al., 2003; Winding et al., 2005; Bécaert & Deschênes, 2006). As the bacterial community structure (molecular community profile) reacts rapidly to soil hydrocarbon contamination (Paper II), community fingerprinting seems a promising indicator also for pollution-induced community disturbance. Interestingly, with plants such a passive *in situ* ecotoxicity test has already been applied (Paton et al., 2005). The direction (weakening or restoring community) and relative size of the pollutant effect can be evaluated by comparison to an undisturbed reference (Paper II; Banning et al., 2011). The ecological status of the soil is completely restored once the indigenous microbial community no longer significantly differs from uncontaminated control soil community. The major challenge is the large natural temporal and spatial heterogeneity in soils (MacLeod et al., 2001): bacterial community structure will respond not just to oil but every other changing parameter too. Against what reference can the community structure be compared if no otherwise identical clean control soil is available and/or long-term temporal monitoring is difficult? One solution could be parallel profiling of RNA-based presently active soil communities and DNA-based present+past communities. In Paper IV, constrained ordination revealed that the surface soil present communities resembled past communities not at the same contamination level but in significantly cleaner soil. Such a result could indicate that the aerated soils at the landfarming field were already recovering from the contamination disturbance, thanks to aerobic hydrocarbon biodegradation.

Interestingly, a decrease in organic substrates and/or microbial biomass does not necessary cause a decrease in operational bacterial diversity (Hirsch et al., 2009). On the other hand, a relative competitive advantage due to

substrate input will nearly unavoidably affect bacterial evenness negatively (Paper I), and increased toxicity can also decrease diversity (Paper III). A decrease in observed diversity can have negative ecological effects on community functioning, no matter whether the cause is selective toxicity or favouring, through complex interactions and competition for nutrients (Allison & Martiny, 2008). Community evenness in general seems preferable, since it indicates a lower disturbance (Torsvik et al., 1996). However, it is probably not sensible to compare apparent diversity in very differing samples – even in the surface and subsurface soils in Paper IV – as the diversity indices are purely operational and completely technique-dependent measures. That being said, the comparison of DNA and RNA-based community evenness in the same sample might be useful for the assessment of temporal contamination response. In Paper IV, the higher evenness of the present bacterial community compared to the past community supported the results of constrained ordination, suggesting that the surface soil microbial ecological status was recovering.

Besides the difficulty of reliably measuring soil microbial community diversity, it could be asked what the ecological meaning of soil microbial α-diversity (diversity within one studied unit, e.g., soil sample) actually is. In the ecology of flora and fauna, medium disturbance has been observed to support the highest diversity (Ollivier et al., 2011). This theory seems inapplicable to bacteria and hydrocarbons, since medium level contamination will specifically favour degrader taxa, leading to a decrease in the apparent diversity (Paper II). In general, diversity is the basis of functional redundancy and resistance as well as community resilience (Winding et al., 2005; Bécaert & Deschênes, 2006). Species richness should also buffer adverse consequences: in a highly diverse community, the relative impact of the loss of few most sensitive taxa is smaller (Allison & Martiny, 2008). Due to the extreme genetic diversity and high redundancy of soil microbial communities, a minor decrease in richness *per se* should not be detrimental; Bécaert & Deschênes (2006) claim that the loss of microbial diversity due to soil contamination should risk only very specialised functions. In addition, in soil microbial ecology, a lack of richness and the disappearance of taxa or function are not easy to prove. Even very intensive oil contamination is highly unlikely to reach every single micro-environment – the soil structure thus secures the preservation of genetic metabolic potential. However, the presence of only a very small number of microbes capable of executing a special function may result in an extremely slow start of the process, especially if the coordinated activity of consortia is required. In liquid cultures this may take hundreds of days (Gray et al., 2011), but much longer times should be expected in soil where the relevant microbes can be spatially isolated.

Exploring community composition, i.e., the classification of the abundant or changing taxa, may also improve the ecological inference on the quality of contaminated soil. Accumulating literature can help assign putative

ecophysiological roles to the identified taxa, especially to the intensively studied bacteria. However, taxonomy or even the presence of functional genes cannot reliably predict realised functional activity (Prosser & Nicol, 2008; Mußmann et al., 2011). In addition, the search for indicator taxa which are globally abundant but especially sensitive to hydrocarbon contaminants has so far resulted in little success. An increase in the relative abundance of groups regarded r-strategic over K-strategists – rapidly reacting utilisers of readily available substrates, or slower persistently enduring organisms, respectively (Fierer et al., 2007) – might serve as an indicator of stress and/or hydrocarbon availability (Paper II; Torsvik et al., 1996; MacLeod et al., 2001; Margesin et al., 2003). Classification may also help to identify problems in biodegradation, the detection of anaerobic taxa even in the surface soil on the landfarming field as an example (Paper IV). Nevertheless, one should not jump to conclusions, because our knowledge on which degraders can be efficient in which conditions is far from complete. A good example of this was presented in Paper II where *Aquabacterium* was identified as the possible fuel oil degrader, regardless of the fact that Betaproteobacteria have not been earlier associated with alkane degradation. However, the presence of indigenous microbial groups that have been formerly connected to hydrocarbon degradation in soil indicates catabolic potential, especially if the taxa are shown to be relatively active though DNA-RNA comparison (Paper IV). Unfortunately, definitive linking of metabolic activity of a taxon to realised contaminant degradation rate *in situ* is extremely difficult.

4.2 MICROBIAL ECOLOGY IN CONTAMINATED SOIL RISK ASSESSMENT

Microbial ecological indicators are nowadays included in or introduced to several native soil quality monitoring programs (Winding et al., 2005; Ritz et al., 2009). However, in the field of contaminated soil risk assessment and management, microbial ecology has no established role. It may be asked whether microbial ecological indicators even should be included in the ecological risk assessment process for oil-polluted areas: the classical soil quality indicators are not directly applicable, and toxicological responses are not straightforward enough to interpret. However, the intrinsic relevance of microbial ecological indicators to soil quality and functioning is undeniable, and they sensitively summarise the collective chronic stress from the multitude of hydrocarbon contaminants and metabolites that are difficult to quantify with chemical analyses (Paper III; Brassington et al., 2007). In ecological risk assessment, soil microbiological variables indicate not just contaminant effects on soil microflora but also on higher organisms and plant production (MacLeod et al., 2001). In the tiered triad approach of ecological risk assessment that evaluates chemical, (eco)toxicological and

ecological lines of evidence together (MacLeod et al., 2001; Winding et al., 2005, Brassington et al., 2007), soil microbial ecological indicators provide information on both ecotoxicology and ecology.

In the Finnish environmental legislation, risk-based assessment of a contaminated site requires the estimation of risk to people (health risk), surroundings (spreading risk) and nature (ecological risk) (Ympäristöministeriö, 2007). The protection of human health and the prevention of spreading are always the principal aims (Brassington et al., 2007), but better consideration of also the ecological risk has lately been discussed actively in both national (MUTKU-päivät, Soili-seminaarit) and international (NORDROCS) meetings. Interestingly, Fränzle (2006) claimed that the inability of severely chemically stressed soil to perform its ecological functions is realised as harmful fluxes to neighbouring ecosystems. Thus the weakened soil ecological status is linked to spreading risk through impaired soil ecosystem services, specifically the regulating (buffering) functions. On the other hand, if soil pollutants do not seem to be bioavailable to soil microbes, which are in constant and direct contact with them and have specific strategies to enhance the uptake, chances of high bioavailability and risk to human receptors seem unlikely.

Bécaert & Deschênes (2006) suggested that investigating soil health through its microflora could guide remediation actions. Microbial ecological considerations could be better included in risk assessment and management decision making, starting from the initial decision on how – and whether – the contaminated soil masses should be restored (Siciliano & Roy, 1999; MacLeod et al., 2001). In addition, the ecological burden of the typically energy-intensive and potentially destructive remediation activities must also be considered (Grotenhuis & Rijnaarts, 2011). The majority of soil-based ecosystem services are provisioned by the thin layer of surface soil, which is a practically unrenewable natural resource (Banwart, 2011). To simplify, this leads to the conclusion that the soil that functions the poorest is the soil that is removed. Excavation and disposal of slightly or moderately contaminated masses should thus be reconsidered from a wider perspective of ecological sustainability. Clean sand or gravel material brought in to substitute for the disposed contaminated soil tons, as well as a compost-based organic layer possibly spread on top, have no risk of subjecting people or surrounding nature to hydrocarbon exposure. However, such materials show also generally poor stability, and likely reduced ability to provide various ecosystem services at least for some period of time.

Further on, Paton et al. (2005) emphasised that a holistic approach is needed also for the assessment of remediation results. Especially in the case of aged hydrocarbon contamination, efficiency of remediation should be monitored not as reduction in TPH but through reduced chemical mass and toxicity (Paper III; MacLeod et al., 2001; Brassington et al., 2007). Moreover, if the contaminated masses are remediated *in situ* (Brassington et al., 2007; Grotenhuis & Rijnaarts, 2011,) or will be used as soil at some other

location afterwards, it seems justified to evaluate their quality as soil, not as waste material. This again argues for the relevance of microbial ecological indicators, and the consideration of microbial ecological responses also in the selection of remediation method. Strong physicochemical techniques, such as chemical oxidation, may have long term detrimental effects on soil quality (Palmroth et al., 2006; Grotenhuis & Rijnaarts, 2011). On the other hand, bioremediation techniques aim at enhancing the activity of indigenous microbial hydrocarbon degraders (Brassington et al., 2007; Grotenhuis & Rijnaarts, 2011), typically concomitantly improving the general microbial ecological status of the soil. Bioremediation could thus, for a majority of hydrocarbon-contaminated soils, be that ecologically sustainable and green remediation approach that Grotenhuis & Rijnaarts (2011) call for, especially if monitored with appropriate soil microbial ecological quality indicators.

The results of Papers I-IV, together with the literature survey carried out for this thesis compilation, leads me to suggest the following soil microbiological quality indicators for the ecological risk assessment of hydrocarbon-contaminated soils:

<u>Soil status: affected by pollution</u>

1. Chronic decrease in basal respiration and/or microbial biomass regardless of increase in soil carbon
   = severe detrimental effects of oil pollution on soil ecology

2a. Decrease in bacterial community evenness (spatial or temporal response to hydrocarbon increase)
   = oil bioavailable and potentially toxic

2b. Increase in the ratio of hydrocarbon degraders to heterotrophs (spatial or temporal response to hydrocarbon increase)
   = oil bioavailable and potentially toxic

<u>Soil status: recovering after pollution</u>

3. RNA-based bacterial community fingerprints systematically resemble DNA-based fingerprints in soil of not the same but lower contamination level
   = soil ecological status likely already recovering

4a. Bacterial community evenness stabilises after increasing (spatial or temporal response to hydrocarbon decrease)
   = soil ecological status (nearly) recovered

4b. Ratio of hydrocarbon degraders to heterotrophs stabilises after decreasing (spatial or temporal response to hydrocarbon decrease)
   = soil ecological status (nearly) recovered

<u>Soil status: unaffected by pollution or restored after pollution</u>

5. Bacterial community structure does not significantly differ from clean reference soil, and contamination level does not correlate with bacterial community changes
   = soil ecological status completely recovered

Although these indicators seem to be the most promising ones both theoretically and operationally, their use in contaminated soil risk assessment requires further validation. Comparison of DNA- and RNA-based community fingerprints for Indicator 3 should still be tested with an experiment where an appropriate uncontaminated reference soil is included, and the remediation endpoint is (legislatively) clean soil. I also currently assume that practically any relevant and sufficiently robust quantification method (molecular or culture-based) should be applicable for the enumeration of hydrocarbon degraders for Indicators 2b and 4b, but this requires further testing. Fortunately, such data should be well accessible from earlier literature.

The relevance of results on soil microbial ecological status that are produced by analysing homogenised mixed samples might also be questioned. Soil prokaryotes live in widely differing microniches, where the access or exposure to contaminants as well as other substances (water, oxygen, nutrients) is very varied. The response observed in the scale of gram or kilogram soil samples may precisely represent very few individual organisms. However, the suggested indicators do, in average, reflect the authentic situation *in situ*. In addition, provision of observable ecosystem services, or toxicity to multicellular organisms, generally neither take place in the micrometer scale habitats but in larger soil masses. For in depth study of the constraints of hydrocarbon biodegradation (bioremediation), techniques investigating the microscale *in situ* communities may be necessary. However, for ecological risk assessment purposes they seem generally redundant and unpractical.

5 CONCLUSIONS

Increasing demands for land use and the EU soil directive currently under preparation require more sustainable management of soils, including contaminated sites. Profound understanding of soil microbial ecological status is important in polluted site risk assessment as well as in the development of green remediation approaches. In the case of crude oil or oil product contamination, such understanding has so far been hindered by the fact that the commonly recommended indicators for soil quality typically respond positively to added hydrocarbons.

This thesis evaluated the suitability of selected quantitative and qualitative soil microbial variables for monitoring of the ecological effects of soil hydrocarbon contamination and biodegradation. Soil respiration and microbial biomass alone were found to be inappropriate indicators for the deleterious effects of oil: such measures reflecting the redundant heterotroph community respond negatively only to extremely heavy and/or repeated contamination. However, changes in the ratio of degrader numbers to total community should accurately reflect changes in hydrocarbon bioavailability – once the decrease in this quotient ceases, possibly remaining oil is no more available to the degraders or toxic to other soil biota.

Qualitative properties of the soil bacterial community, especially community evenness and structure, respond readily to contamination and restoration. The interpretation of these multivariate data derived indicators has been regarded challenging unless appropriate clean reference soil is available and/or there is a possibility for long-term monitoring (over years). However, an increase in evenness can alone be regarded as a sign of community recovery and stabilisation after oil contamination; evaluating this might even be possible without references, by comparing the pools of soil DNA (including historical community) and RNA (active present community). When samples otherwise similar but with different hydrocarbon concentrations are available, the DNA-RNA comparison can be combined to constrained multivariate ordination to see if the past and present communities reflect contamination level differently.

Unlike previously suggested, the numbers of ammonia oxidisers or archaea were not especially sensitive to oil, and are thus an inappropriate indicator for contamination disturbance. However, microbial community composition, for example, a high relative abundance of active aerobic or anaerobic degrader populations, may help estimate the general relevance of hydrocarbons as substrates for the soil heterotrophic community.

In practice, the use and interpretation of microbial ecological variables in the assessment of oil contamination and degradation typically still requires case-specific expert evaluation. Since soil microbial properties reflect so readily not just hydrocarbon pollution but also multiple other factors,

establishing optimal values or reference communities seems impossible even in theory. As the environmental relevance of soil ecotoxicological assay has been claimed to be inversely correlated with the difficulty of the procedure (Paton et al., 2005), it is a great challenge for microbial ecologists to identify, refine and develop practical hydrocarbon bioavailability indicators. This issue is exacerbated by the fact that the field of environmental microbiology in general is very excited about novel technically challenging methods that provide unforeseen chances for discovery, but may not fill the requirements of reproducibility and cost-effectiveness needed in routine environmental quality monitoring.

The results of this work demonstrate that there are still good chances to streamline many microbiological methods deemed relevant but difficult: turbidity-based MPN enumeration of degraders and LH-PCR assessment of community changes with simplified curve-based data analysis are good examples. These methods broadcast technical simplicity and robustness, compared to the currently more commonly used alternatives qPCR and T-RFLP/pyrosequencing. To benchmark the value of soil microbial ecological monitoring to politicians, consultants and land-owners, the costs must be carefully justified, striving for the most economical analyses that can answer the questions relevant for environmental risk management.

With regard to hydrocarbon-contamination, microbial ecological indicators will never replace all chemical oil and soil analyses, nor solve the challenges of representative environmental sampling at such heterogeneous sites. However, they can be practical and intrinsically relevant technical and theoretical tools for ecological risk assessment. Thus, consideration of microbial ecology can benefit ecologically informed and sustainable – ecosophisticated – management of oil-polluted lands.

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